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Cytochrome bc_1 Complexes of Microorganisms

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INTRODUCTION

The cytochrome bc_1 complex is an oligomeric membrane protein complex which transfers electrons from a relatively low-potential quinol to a *c*-type cytochrome or plastocyanin and deposits four protons on the electropositive side of the membrane per pair of electrons transferred to two molecules of cytochrome *c*. The reaction catalyzed by the bc_1 complex is thus described by the equation below, where the subscripts n and p designate negative and positive surfaces of the membrane, and c_{ox} and c_{red} refer to oxidized and reduced cytochrome:

$$QH_2 + 2H^+_n + 2\ c_{ox} \rightarrow Q + 4H^+_p + 2\ c_{red}$$

This proton motive activity of the bc_1 complex converts the available free energy of the electron transfer reaction into an electrochemical proton gradient across the membrane in which the complex is asymmetrically located. In bacteria the cytochrome bc_1 complex is localized in the plasma membrane, and the vectorial translocation of protons is such that the periplasmic side of the plasma membrane is electropositive. In mitochondria the bc_1 complex is localized in the inner mitochondrial membrane, and the high protonic potential is formed on the cytosolic side of the membrane.

In this review I will discuss the cytochrome bc_1 complexes of microorganisms, with emphasis on those in which the physiological functions, subunit compositions, and molecular genetics of the bc_1 complex are best understood. I have occasionally used the term cytochrome bc_1 complex generically to also include cytochrome *bf* complexes. In instances when I have distinguished between complexes of the bc_1 and *bf* types, I have defined the *bf* complexes as those in which cytochrome *b* is split into two subunits, encoded by two genes, and the accompanying cytochrome in the membranous complex is cytochrome *f*.

I will summarize what is known of the phylogenetic distribution of the bc_1 complexes and discuss the functional relationships of the bc_1 complexes to other electron transfer complexes under various growth conditions. I will also discuss the composition and structure of the bc_1 complex, the mechanism of electron transfer and proton translocation, and the differences between the bacterial and mitochondrial complexes. In discussing these aspects of the bc_1 complex, I will also highlight several unanswered questions which, in my view, are especially timely.

There are several additional reviews on related subjects which I found especially helpful and which the reader may wish to also consult. These include reviews on bacterial electron transport chains (5), pathways of electron transfer in anaerobic respiration and photosynthesis in members of the family *Rhodospirilliceae* (50, 51), electron transport in green photosynthetic bacteria (15), sulfur oxidation by phototrophic bacteria (25), energy transduction in anoxygenic photosynthesis (45), genetics of the cytochrome bc_1 complex in purple photosynthetic bacteria (54, 135), and transcriptional regulation of cytochrome gene expression in *Saccharomyces cerevisiae* (53).

OCCURRENCE AND PHYSIOLOGICAL FUNCTION OF THE CYTOCHROME bc_1 COMPLEXES

Phylogenetic Distribution of Cytochrome bc_1 Complexes

The cytochrome bc_1 complex is the most common of the energy-transducing electron transfer complexes. Cytochrome bc_1 complexes have been isolated from the plasma membranes or derivatives thereof of both gram-negative and gram-positive bacteria, bacteria which use oxygen, nitrogen, and sulfur compounds as terminal electron acceptors, from oxygenic and anoxygenic photosynthetic bacteria, and from mitochondria of lower and higher eucaryotes. A very similar cytochrome *bf* complex occurs in algae and the chloroplasts of higher plants.

The bc_1 complex is common, but not universal, in procaryotes. The single most reliable indication of a bc_1 or *bf* complex is the presence of a Rieske-type iron-sulfur protein, although an electron paramagnetic resonance (EPR) signal similar to that of the Rieske protein is also associated with phthalate dioxygenase found in some bacteria (91). There are numerous *b*- and *c*-type cytochromes unrelated to the bc_1 complex, especially in bacteria, and there are various quinol oxidases which do not involve the bc_1 complex. Antimycin and myxothiazol, although apparently specific inhibitors of all bc_1 complexes, are frequently not cytotoxic to microor-

TABLE 1. Procaryotes containing cytochrome bc_1 complexes

Organism	Characteristics	Reference(s)
Paracoccus denitrificans	Denitrifying, closely related to purple photosynthetic bacteria	180
Pseudomonas stutzeri	Denitrifying pseudomonad	89
Bradyrhizobium japonicum	Nitrogen fixing	146
Pseudomonas cichorii	Phytopathogen, fluorescent, obligate aerobe	187
Rhodobacter capsulatus	Photosynthetic, purple, nonsulfur	99
Rhodospirillum rubrum	Photosynthetic, purple, nonsulfur	177
Rhodobacter sphaeroides	Photosynthetic, purple, nonsulfur	99, 183
Rhodopseudomonas viridis	Photosynthetic, purple, nonsulfur	81, 177, 178; cf 54.
Rhodobacter sulfidophilus	Photosynthetic, purple, sulfur	26
Chromatium vinosum	Photosynthetic, purple, sulfur	46, 57
Chlorobium limicola	Photosynthetic, anoxygenic, anaerobic, green, sulfur	72
Chloroflexus aurantiacus	Photosynthetic, anoxygenic, thermophilic, facultative aerobic, gliding green	177, 179, 188
Heliobacterium chlorum	Photosynthetic, anoxygenic, green	Liebl et al., in press
PS3	Gram-positive, thermophilic bacillus	93
Bacillus alcalophilus	Gram-positive, obligate alkalophilic	96
Anabaena variabilis	Photosynthetic, filamentous cyanobacterium	86
Nostoc spp.	Photosynthetic, filamentous cyanobacterium	79

ganisms which have alternate electron transfer pathways in addition to a bc_1 complex, nor do they effectively inhibit the *bf* complex. Consequently, lack of sensitivity to these bc_1 inhibitors does not preclude the involvement of a bc_1 or *bf* complex in the electron transfer pathways of the microorganism.

In Table 1 I have listed procaryotes that have been shown to contain a bc_1 or *bf* complex. The complexes have not been purified from many of these species. Therefore, I have taken as evidence for the existence of a bc_1 or *bf* complex the presence of a Rieske-type iron-sulfur protein along with membranous *b*- and *c*-type cytochromes, the isolation of genes encoding these proteins characteristic of the bc_1 or *bf* complexes, or the demonstration of antimycin- or myxothiazol-sensitive electron transfer.

The bc_1 complexes have been purified and characterized to various degrees from three photosynthetic purple nonsulfur bacteria and from *Paracoccus denitrificans*, a closely related, nonphotosynthetic bacterium. In Table 2 I have listed the subunit compositions and turnover numbers of the bc_1 and *bf* complexes which have been purified to date. The activities of the complexes from PS3, *Anabaena variabilis*, and *Neurospora crassa* seem unusually low. Whether these low activities are intrinsic properties of these complexes or whether the complexes have been damaged during purification is not known.

TABLE 2. Subunit compositions and activities of procaryotic and eucaryotic cytochrome bc_1 complexes

Organism	No. of subunits	T_n (s^{-1})[a]	Reference
Procaryotes			
Paracoccus denitrificans	3	500/700	180
Rhodobacter capsulatus	4	64/70	99
Rhodospirillum rubrum	3	234	177
Rhodobacter sphaeroides	4	128/180	99
PS3	4	50	93
Anabaena variabilis	4	20	86
Eucaryotes			
Saccharomyces cerevisiae	9	220/305	99
Neurospora crassa	9	20	143

[a] Where two turnover numbers (T_n) are reported, the number before the slash is the turnover number of the purified complex and the number after the slash is the turnover number of the washed membranes after dispersal with dodecyl maltoside. Where a single turnover number is reported, it is for the purified complex.

The *fbc* operon encoding the Rieske iron-sulfur protein, cytochrome *b*, and cytochrome c_1 was first cloned and sequenced from a photosynthetic bacterium (34, 36, 55). Originally it was thought that this clone was from *Rhodobacter sphaeroides* (55). Subsequently, when the operon was cloned and sequenced from *Rhodobacter capsulatus* and also cloned and partially sequenced from a bona fide *R. sphaeroides* strain, it was realized that the original clone was isolated from *R. capsulatus* (36). An operon encoding the bc_1 complex has also been cloned and sequenced from the denitrifying bacterium *P. denitrificans* (92), and from the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (146).

A cytochrome *bf* complex has been purified from the cyanobacterium *Anabaena variabilis* (86), and the genes encoding the four subunits of the *bf* complex have been cloned and sequenced from a second cyanobacterium, *Nostoc* spp. (78, 79). Interestingly, the complex purified from the gram-positive bacillus, PS3, appears to be a cytochrome *bf* (93), rather than bc_1 complex, establishing the precedent that the *bf*-type complex is not restricted to chloroplast-containing species. The gram-positive *Bacillus alcalophilus* contains a Rieske-type iron-sulfur protein and cytochromes of the *b* and *c* type (96), but the electron transfer complex has not been purified from the alkalophile, and it is not known whether it is a bc_1 or *bf* complex. Likewise, it is not known whether the occurrence of a *bf* complex extends to other gram-positive bacteria, such as *Bacillus subtilis*.

The photosynthetic purple sulfur bacterium *Chromatium vinosum* (46, 57), the photosynthetic green sulfur bacterium *Chlorobium limicola* (84), and the photosynthetic gliding green bacterium *Chloroflexus aurantiacus* (177, 188) exhibit EPR signals characteristic of the Rieske iron-sulfur protein. The EPR signal of the Rieske iron-sulfur protein is essentially identical in bc_1 and *bf* complexes, and whether these species contain complexes of the bc_1 or *bf* type is not known (however, see below).

Heliobacterium chlorum, a recently discovered gram-negative, anoxygenic, photosynthetic bacterium, contains a Rieske iron-sulfur protein which is sensitive to 2,5-dibromo-3-methyl-6-isopropylbenzoquinone and stigmatellin (U. Liebl, W. Rutherford, and W. Nitschke, FEBS Lett., in press). Since this bacterium resembles gram-positive bacteria in numerous respects, it would be interesting to know whether it contains a complex of the bc_1 or *bf* type.

The cytochrome bc_1 complex is an obligatory component of the respiratory chain of mitochondria and is constitutive in most eucaryotes, with the exception of *S. cerevisiae*, in which the respiratory chain proteins are subject to catabolite repression by glucose under conditions of fermentative growth (49). The bc_1 complexes of both *S. cerevisiae* (99, 137, 138) and *N. crassa* (170) have been purified and extensively characterized (Table 2), and the genes encoding all of the subunits of the *S. cerevisiae* complex have been cloned and sequenced (for a review, see reference 40).

Parasites such as *Trypanosome brucei* and *Leishmania tartentolae* contain bc_1 complexes which are functional in the procyclic insect vector, but absent from the bloodstream forms of these organisms (108). The mechanism by which the bc_1 complex is repressed when the parasite is in the bloodstream is not known. Using a procedure similar to that used to purify the bc_1 complexes from various, phylogenetically diverse species (99), Berry and Simpson have isolated and partially characterized the bc_1 complex from *L. tartentolae* (E. A. Berry, personal communication). Although the exact subunit composition is still under investigation, preliminary results indicate that the complex from this parasite resembles those from other eucaryotes in containing several, non-redox-group-containing supernumerary polypeptides (see below).

This survey gives some indication of the extensive phylogenetic distribution of the bc_1 and *bf* complexes. At the same time, it should be noted that *Escherichia coli* does not contain a bc_1 complex, and hence the application of genetics and molecular biology to the study of this energy-transducing complex is a relatively recent undertaking. Archaebacteria apparently do not contain a bc_1 or *bf* complex, and the point at which the bc_1 or *bf* complex appeared in evolution is not known.

Hauska et al. (68) have suggested that quinol-cytochrome *c*/plastocyanin oxidoreductases are an "ancient, conservative core of electron transport," having originated with the green sulfur bacteria, which, in their view, may be the oldest phototrophic organisms and the phylogenetic progenitors of the cyanobacteria and purple photosynthetic bacteria. Knaff and Buchanon (83) found that reduction of NAD^+ by sulfide in *Chlorobium limicola* is inhibited by antimycin, which suggests that this primitive organism contains a complex of the bc_1 type. Complexes of the bc_1 type may thus have appeared in microorganisms before complexes of the *bf* type. This would suggest that the gene for cytochrome *b* in the chloroplast *bf* complex and gram-positive bacteria split into two proteins as it evolved, which together retain a high degree of structural homology to the *b* cytochromes of mitochondria and other gram-negative procaryotes, in which cytochrome *b* is a single protein (see below). It would be especially informative to purify the putative bc_1 complex from *Chlorobium* spp. and to extend similar efforts to other phylogenetically diverse, primitive organisms.

Relationship of Cytochrome bc_1 Complexes to Alternate Electron Transfer Pathways

The role of the bc_1 complex in cellular energy transduction and its relationship to other electron transfer complexes vary among procaryotes. In addition, there is no correlation between photosynthetic and nonphotosynthetic organisms and the involvement of a bc_1 or *bf* type electron transfer complex. However, two generalities seem to apply. The first is that all photosynthetically driven cyclic electron transfer obligatorily involves either a bc_1 complex, as in the examples of *R. sphaeroides* and *R. capsulatus*, or *bf* complex, as in cyanobacteria.

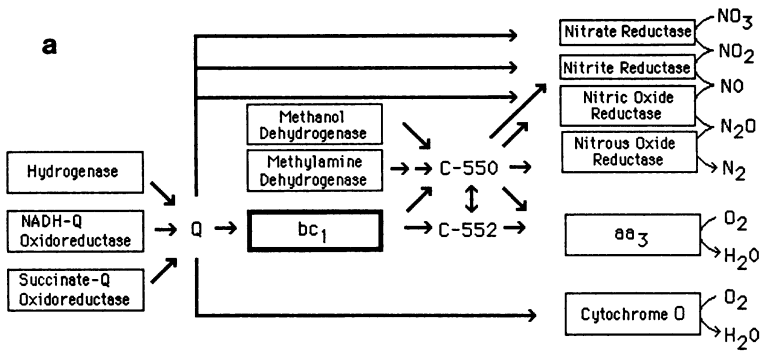
The second generality is that all bacteria which contain a bc_1 complex possess at least one alternate ubiquinol oxidase pathway, which circumvents the bc_1 complex when the bacteria are grown nonphotosynthetically, under either aerobic or anaerobic conditions. These alternate oxidases lack the energy-transducing (proton-translocating) activity of the bc_1 complex and thus support growth of the bacteria somewhat less effectively. However, as in the case of the L-lactate/cytochrome *c* oxidoreductase of *S. cerevisiae* (see below), the emergence of the alternate oxidase pathways may have provided these bacteria with a protection against competing microorganisms which produce toxins acting on the bc_1 complex, such as myxothiazol and antimycin.

Myxobacteria (58), and the basidiomycetes *Strobilurus* (3) and *Oudemansiella* (4) spp. produce toxins, myxothiazol, strobilurin A, and oudemansin, which inhibit the bc_1 complex. Whether these microorganisms contain bc_1 complexes and, if so, whether these complexes are inherently resistant to these toxins is not known (9, 145). An alternative explanation for the inherent resistance of such organisms to bc_1 toxins is that they possess complexes of the *bf* type. Splitting of cytochrome *b* into two proteins in the *bf* complexes appears to be universally associated with growth resistance to antimycin and with diminished sensitivity to myxothiazol, strobilurin, and oudemansin. However, it appears that *Strobilurus* spp. contain a bc_1, and not *bf*, complex (G. von Jagow and H. Scägger, personal communication). This suggests that these toxin-producing microorganisms rely on an alternate, toxin-insensitive oxidase pathway for growth (see below).

The scheme in Fig. 1a, based on those proposed by Ferguson and co-workers (27, 50, 117, 124), summarizes the relationship of the bc_1 complex to other electron transfer complexes in *P. denitrificans*, a nonfermenting, denitrifying soil bacterium capable of autotrophic growth on H_2 and CO_2 or heterotrophic growth on a variety of carbon sources. When grown aerobically, *P. denitrificans* elaborates a respiratory chain superficially so similar to that of mitochondria that this organism is frequently described as resembling a free-living mitochondrion (75).

The relationship of the bc_1 complex to other electron transfer complexes in *P. denitrificans* is, in many respects, a paradigm for nonphotosynthetic, facultative anaerobes which contain a bc_1 complex. Flavin- or NAD-linked dehydrogenases reduce ubiquinone to ubiquinol, as does hydrogenase (Fig. 1a). A key feature of these bacterial electron transfer systems is that ubiquinol is then oxidized by one or more alternative routes. Under anaerobic, denitrifying conditions, ubiquinol is the low-potential electron donor for nitrate reductase (32, 90, 118), bypassing the bc_1 complex and *c*-type cytochromes in an antimycin- (75) and strobilurin (87, 118)-insensitive reaction. Further reduction of the nitrogen acceptor, via nitrite and nitrous oxide reductase, involves the bc_1 complex and the *c* cytochromes, as indicated by sensitivity of these reactions to bc_1 complex inhibitors (17, 87, 89; however, see 51).

This branching of electron transfer pathways must occur at the level of ubiquinol oxidation, and not within the cytochrome bc_1 complex. There is no electron transfer from one bc_1 complex to another on a physiologically relevant time scale. This can be inferred from the fact that in experiments examining effects of inhibitors on pre-steady-state reduction of the cytochromes, inhibition of cytochrome c_1 reduction by myxothiazol (167) and inhibition of cy-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Methanol Dehydrogenase
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Nitrate Reductase
Nitrite Reductase
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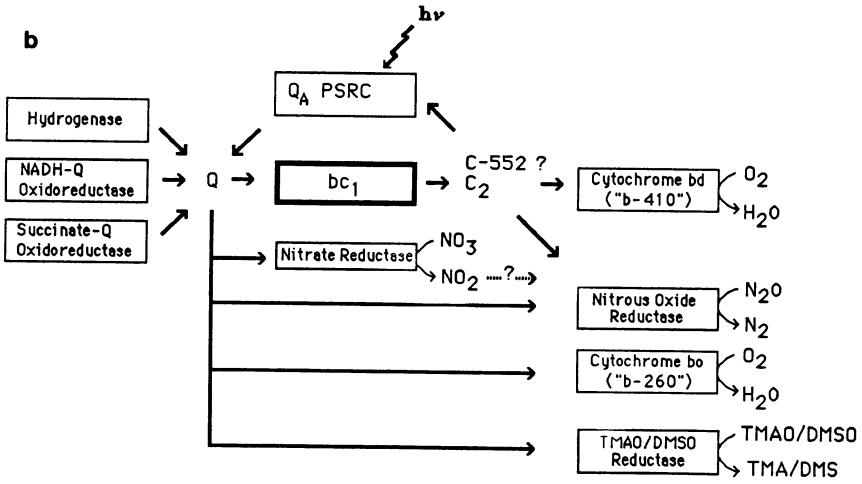
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bc1
C-552 ?
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Nitrate Reductase
NO3
NO2
Nitrous Oxide Reductase
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TMAO/DMSO Reductase
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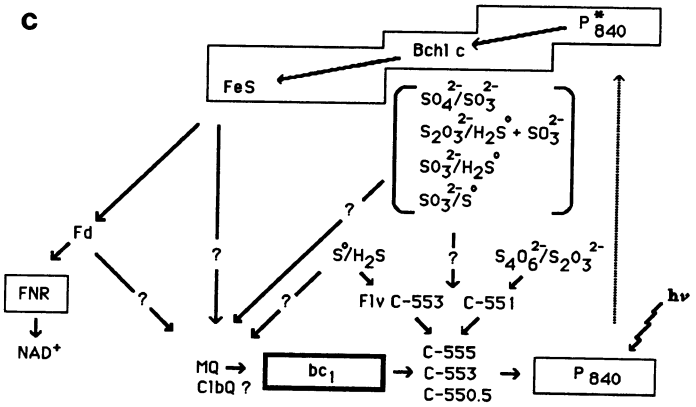
N2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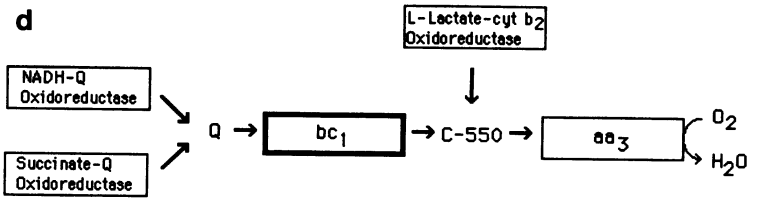
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tochrome *b* reduction by antimycin in the iron-sulfur protein-depleted complex (47) titrate as linear curves. If electron transfer from one complex to another were possible, one would expect that a fractional equivalent of inhibitor would fail to block electron transfer through the complex, since the lateral electron transfer from one complex to another would effectively bypass the inhibitor. This in turn would give rise to a "lag" in the titration curves. Such is not the case.

If *P. denitrificans* is grown in the presence of oxygen, ubiquinol is oxidized by the cytochrome aa_3-type oxidase via the bc_1 complex. Electron transfer between the bc_1 and aa_3 complexes preferentially uses a membrane-bound cytochrome *c*-552 (13), although at least partial redox equilibration with the periplasmic cytochrome *c*-550 does occur. This preferential utilization of *c*-552 appears to be due to the transient association of the cytochrome bc_1 and cytochrome aa_3 complexes, along with cytochrome *c*-552, to form a ubiquinol oxidase super complex (13). A similar association of electron transfer complexes occurs in the gram-positive thermophile PS3 (141), and Joliot et al. (76) obtained kinetic evidence for the formation of a super complex between the cytochrome bc_1 complex, cytochrome c_2, and the photosynthetic reaction center in *R. sphaeroides*. Such ternary complexes may be more commonly involved in bacterial electron transfer chains than is currently appreciated.

Attempts to demonstrate the formation of a comparable ubiquinol oxidase complex in mitochondria were unsuccessful (13). The apparent lack of any high-affinity association between the bc_1 and aa_3 complexes in mitochondria is consistent with the view that these electron transfer complexes are independently laterally mobile in the inner mitochondrial membrane and that the rate of respiration is diffusion controlled (61).

The extent to which an *o*-type cytochrome acts as terminal acceptor under aerobic conditions is not known and probably varies with growth conditions and different strains of *P. denitrificans* (118, 132, 160). In addition to allowing the bacteria to survive exposure to bc_1 complex toxins, the alternate quinol oxidase pathway probably provides a proton motive release valve. As the electrochemical proton potential becomes higher with diminished ATP consumption, the membrane potential and pH gradient components of the proton motive force would retard electron flow through the bc_1 complex. Under these conditions, reducing equivalents which would otherwise accumulate in ubiquinol would be diverted through the pathway of lower proton motive resistance to the *o*-type cytochrome.

In addition to the bifurcating ubiquinol oxidase pathway, *P. denitrificans* has a periplasmic methanol dehydrogenase (Fig. 1a), which utilizes a bound pyrrolo-quinoline quinone cofactor (44), rather than NAD or flavin, and reduces cytochrome *c*-550 directly (1). Deposition of protons from methanol oxidation into the periplasmic space contributes to the proton motive force, but this route of electrons to the terminal oxidase also occurs at the expense of energy transduction by the bc_1 complex, thus accounting for lower growth rates on methanol. Likewise, methylamine dehydrogenase is located in the periplasmic space and apparently bypasses the bc_1 complex, transferring electrons to cytochrome *c*-550 via the blue copper protein, amicyanin, and cytochrome *c*-551 (159).

Photosynthetic bacteria obligatorily use the cytochrome bc_1 complex for light-driven cyclic electron transfer. The scheme in Fig. 1b summarizes the relationship of the bc_1 complex to other electron transfer complexes in *R. capsulatus*. When *R. capsulatus* is grown phototrophically the cytochrome bc_1 complex oxidizes ubiquinol and reduces a *c* cytochrome, which is then reoxidized by the photosynthetic reaction center complex in a light-driven reaction spanning approximately 1 V. The reaction center then rereduces ubiquinone to ubiquinol via the special bound quinone, Q_A.

Whereas it is clear that ubiquinol is the reductant for the bc_1 complex in cyclic electron transfer, it is not clear which *c* cytochrome is the oxidant. Cyclic electron transfer is unimpaired in *R. capsulatus* mutants lacking cytochrome c_2 (122). It is possible that this bacteria contains a yet to be discovered membranous cytochrome *c*, analogous to the *c*-552 of *P. denitrificans* (13), and that the reaction center, membranous *c* cytochrome, and bc_1 complex form a ternary super complex, analogous to that in *P. denitrificans*. Alternatively, the cytochrome bc_1 complex and photosynthetic reaction center may transiently associate to form a reaction center–bc_1 binary complex in which electron transfer takes

FIG. 1. (a) Role of the cytochrome bc_1 complex in aerobic respiration and anaerobic denitrification and its relationship to the alternate ubiquinol oxidase pathways in *P. denitrificans*. Ubiquinone is a common electron acceptor pool for the low-potential hydrogenase and oxidoreductases, whereas the *c* cytochromes are a common acceptor pool for the higher-potential methanol and methylamine dehydrogenases, which bypass the cytochrome bc_1 complex. *P. denitrificans* contains numerous cytochromes *c* (159), whose functions are poorly understood, but which appear to have substitutive or overlapping functions in some instances. Only two of the cytochromes *c* are shown. (b) Role of the cytochrome bc_1 complex in cyclic and noncyclic electron transfer in the purple photosynthetic bacterium *R. capsulatus*. The bacterium can grow photosynthetically or chemotrophically, either aerobically or anaerobically, but apparently cannot fix nitrogen owing to the lack of a nitrite reductase, indicated by - - - ? - →. Cyclic photosynthetic electron transfer is driven by the light-harvesting photosynthetic reaction center (PSRC). The uncertain roles of the various *c*-type cytochromes under various conditions of growth are designated by a question mark and discussed in the text. The functions of cytochrome *c'* and the high-potential cytochromes *c*-551 and *c*-557 (121) are not known, and these are not shown. DMSO, Dimethyl sulfoxide; DMS, dimethyl sulfate; TMAO, trimethylamine-*N*-oxide; TMA, trimethylamine. (c) Tentative scheme of cyclic and noncyclic electron transfer in the green photosynthetic sulfur bacterium *Chlorobium limicola* f. *thiosulfatophilum* and related members of the family *Chlorobiaceae*. Question marks indicate uncertainties regarding the oxidants of the various sulfur compounds, and the extent to which the bc_1 complex is an obligatory redox component in the transfer of electrons from the various sulfur compounds to NAD^+. Since the sulfide-quinone redox reaction spans approximately 300 mV, depending on what quinone is the acceptor, it seems likely that currently unrecognized redox carriers participate in electron transfer between sulfide and quinone. Abbreviations: P^*_{840}, light-harvesting bacteriochlorophyll *a* with an absorption maximum at 840 nm; Bchl c, bacteriochlorophyll *c*; Flv C-553, flavocytochrome *c*-553; FNR, ferredoxin-NAD^+ reductase; Fd, ferredoxin; MQ, menaquinone; ClbQ, chlorobium quinone; FeS, iron-sulfur protein. (d) Role of the cytochrome bc_1 complex in the mitochondrial respiratory chain of the budding yeast *S. cerevisiae*. A feature of all mitochondrial respiratory chains is that various dehydrogenases reduce a common pool of ubiquinone, which in turn is oxidized by the cytochrome bc_1 complex. In the scheme shown here, only NADH and succinate dehydrogenases are shown. Yeast mitochondria differ from those of other eucaryotes in that they have a lactate-cytochrome b_2 oxidoreductase which transfers electrons from L-lactate to cytochrome *c*, thus bypassing the cytochrome bc_1 complex. The retention of the bypass in this lower eucaryote is reminiscent of the bypasses of the bc_1 complex common in procaryotes (see panels a and b).

place directly from cytochrome c_1 to the reaction center, obviating the involvement of any mediating cytochrome *c*.

When *R. capsulatus* is grown heterotrophically the functional relationship of the bc_1 complex to other electron transfer complexes is similar to that in *P. denitrificans*. Reducing equivalents can be derived from hydrogenase, or various NAD- or flavin-linked dehydrogenases, all of which reduce the ubiquinone pool. If grown anaerobically, *R. sphaeroides*, but not *R. capsulatus*, can carry out denitrification (Fig. 1b), although there are numerous differences between the nitrogen reductases in *R. sphaeroides* and the better-characterized systems in *E. coli* and *P. denitrificans* (51). The bc_1 complex appears to be involved in the intermediate reduction steps between nitrite and nitrogen, but it is not clear which *c* cytochrome mediates between the bc_1 complex and the terminal reductases (51).

When purple nonsulfur photosynthetic bacteria are grown aerobically in the light, they develop both photosynthetic and respiratory electron transfer chains. The membrane protein complexes of these two electron transfer systems are located in the same continuous cytoplasmic membrane, and there is cross talk between the photosynthetic and respiratory electron transfer chains through the common sharing of the ubiquinone pool, the cytochrome bc_1 complex, and cytochrome c_2. Consequently, continuous or flashing light inhibits respiration.

Two general mechanisms, which do not exclude each other, have been proposed to explain the light-induced inhibition of respiration in nonsulfur purple bacteria. The first is that the electrochemical proton gradient formed by photosynthetic electron transfer inhibits respiration (105). This is essentially the same mechanism which operates during respiratory control in mitochondria, and appears to be applicable in photosynthetic bacteria during continuous illumination. Under these conditions the inhibition of respiration resulting from photosynthetic electron transfer is relieved by uncouplers or ionophores which dissipate the electrochemical proton gradient (105).

The second possible mechanism is that the photosynthetic reaction center and cytochrome oxidase compete for reducing equivalents, such that diversion of electrons to the reaction center retards oxygen uptake. This mechanism appears to operate during short, intermittent flashes of light (94, 161). Initially it was suggested that the reaction center and oxidase compete for reduced cytochrome c_2 (Fig. 1b) (161). Subsequent kinetic tests of this model suggest that the respiratory and photosynthetic electron transfer pathways are linked through the ubiquinone pool and compete for ubiquinol (94). This in turn requires that the population of bc_1 complexes is functionally (but not structurally) heterogeneous, part of the population being transiently associated with the reaction center and the other with the cytochrome oxidase, and that these bc_1 complexes do not equilibrate or cross-react on the time scale of flash-induced electron transfer.

The photosynthetic green sulfur bacterium *Chlorobium limicola* lacks a terminal oxidase of any type and uses reduced sulfur compounds instead of water as a source of reducing equivalents. Although its electron transfer pathways are poorly understood at present, this bacterium appears to rely solely on a cyclic electron transfer system in which an apparently antimycin-sensitive bc_1 complex transfers electrons from quinol to cytochrome *c* (83), presumably establishing an electrochemical proton gradient across the plasma membrane in the process.

A tentative depiction of the cyclic electron transfer system in *Chlorobium limicola* and the postulated role of the bc_1 complex therein is shown in Fig. 1c. Reducing equivalents enter the cycle via a sulfide dehydrogenase, which presumably reduces cytochrome *c* via quinol and the bc_1 complex, thus accounting for antimycin-sensitive reduction of NADP by sulfide. Although at least some sulfide oxidases, which themselves are flavoheme proteins, can reduce cytochrome *c* directly, this reactivity would not account for the reported antimycin-sensitive reduction of NADP by sulfide. The *c* cytochrome is then reoxidized by a light-driven reaction center, which provides low potential reducing equivalents for pyridine nucleotide reduction and regenerates the quinol pool (45).

For other procaryotes thus far characterized, the relationships of the cytochrome bc_1 complexes to other electron transfer complexes are generally variations on the themes illustrated for *P. denitrificans* and *R. capsulatus*. The fluorescent plant pathogen, *Pseudomonas cichorii*, an obligate aerobe, has two divergent electron transfer pathways from ubiquinol to oxygen (187). One of these involves the bc_1 complex and a *c* cytochrome and is antimycin and myxothiazol sensitive; the alternate pathway is insensitive to both of these inhibitors.

A distinguishing feature of the electron transfer systems in *Pseudomonas cichorii* is that although the alternate oxidase appears to be an *o*-type cytochrome, the cytochrome *c* oxidase which services the bc_1 complex is a *b*-type cytochrome. No *a*-type cytochromes could be detected at any stage of bacterial growth (187). The relationships of the bc_1 complexes in *Pseudomonas cichorii* and *R. capsulatus* to the terminal oxidases are similar, in that neither organism has an aa_3-type oxidase, in contrast to *P. denitrificans* and *R. sphaeroides*, both of which elaborate aa_3-type oxidases when grown aerobically.

The nitrogen-fixing *B. japonicum* must have an alternate ubiquinol oxidase pathway, possibly a cytochrome *o* (6), which bypasses the bc_1 complex and allows the bacteria to grow heterotrophically when expression of the bc_1 complex operon is disrupted by a transposon insertion (146). *B. japonicum* contains an aa_3-type cytochrome *c* oxidase when grown aerobically, but which is absent in the anaerobic, nodule bacteroids (6). Although a *B. japonicum* mutant lacking a bc_1 complex can grow in liquid culture, the mutant is both *nif* and *fix* (146). It seems likely that the basis for this phenotype is that nitrogen fixation as measured in liquid culture and some step en route to symbiosis place a higher demand for ATP on the bacterial cell than does noninvasive aerobic growth and that this demand cannot be met by an alternate oxidase pathway which lacks the energy-transducing activities of the bc_1 complex.

The obligatory involvement of the bc_1 complex in symbiosis and nitrogen fixation has important implications for the agricultural economy. Mark Schmitt, in my laboratory, compared the amounts and activities of the cytochrome bc_1 complexes from membranes of wild-type *B. japonicum* and *P. denitrificans*. The content of bc_1 complex was similar in the two organisms. However, the turnover number of the bc_1 complex in membranes from the rhizobia was only 20 s^{-1}, compared with 600 s^{-1} in *P. denitrificans*. In addition, *B. japonicum* is very slow growing, both in liquid culture and in nodules (77).

These observations, and the finding that the bc_1 complex is required for symbiosis and nitrogen fixation, suggest that it may be possible to confer a symbiotic advantage and increase the rate of nitrogen fixation in *B. japonicum* by genetically transferring the operon for the more highly active

cytochrome bc_1 complex from *P. denitrificans* into the nitrogen-fixing bacteria. The likelihood that such enhancement would result from increasing the rate of respiration is confirmed by the finding that a Tn*5* mutant of *Rhizobium phaseoli*, CFN4205, fixed nitrogen at a 33% higher rate when in the nodule form than did the wild-type strain. The enhanced nitrogen-fixing mutant coincidentally exhibited a twofold-higher level of cytochrome *c* oxidase during symbiosis, a fourfold higher level of oxidase when cultured on minimal or complex media, and commensurate increases during respiration (140).

In mitochondria the hydrogen-containing substrates of the respiratory chain are oxidized by specific NADH- or flavin-linked dehydrogenases, which reduce ubiquinone to ubiquinol, a confluence point from which electrons enter the cytochrome-containing portion of the respiratory chain (Fig. 1d). Ubiquinol is then oxidized by the cytochrome bc_1 complex, which transfers two electrons to two molecules of cytochrome *c*.

In most mitochondria there is no route for electron transfer from ubiquinol or from the substrate dehydrogenases to cytochrome *c* oxidase except through the cytochrome bc_1 complex (Fig. 1d). Inhibitors which act on the bc_1 complex are therefore usually cytotoxic to eucaryotes and effective fungicides (4, 136), but are not bacteriocidal owing to the multiple direct routes from ubiquinol to alternate oxidases in most bacteria (see above).

One exception to the obligatory involvement of the bc_1 complex in electron transfer from mitochondrial dehydrogenases to cytochrome *c* oxidase is the L-lactate/cytochrome *c* oxidoreductase of *S. cerevisiae* (Fig. 1d). This enzyme, localized in the yeast mitochondrial intermembrane space, contains flavin and a *b*-type heme. It oxidizes L-lactate specifically and transfers electrons directly to cytochrome *c* (for a review, see reference 40). This bypass of the bc_1 complex is acquired at the expense of a considerable loss of ATP recovery compared with lactate oxidation via the NADH-linked lactate dehydrogenase. However, this bypass may also provide these strains of *S. cerevisiae* a selective advantage over other fungi, when these species cohabit decaying vegetation along with myxospores.

PROTEINS AND GENES OF THE CYTOCHROME bc_1 COMPLEXES

Proteins Containing Redox Prosthetic Groups

All cytochrome bc_1 complexes contain three proteins which contain four redox prosthetic groups. These are cytochrome *b*, which contains two noncovalently bound heme *b* groups (173), cytochrome c_1, which contains a *c*-type heme group covalently attached to the protein through thiol ether linkages (182), and the Rieske iron-sulfur protein, which contains a 2Fe-2S cluster coordinately liganded to two cysteines and two histidines (62, 91). The topographical arrangement of the three redox-containing subunits of the bc_1 complex as found in the plasma membrane of procaryotes such as *P. denitrificans* and *Rhodospirillum rubrum* is shown at the top of Fig. 2.

The cytochrome *bf* complex of chloroplasts, some photosynthetic bacteria, and at least some gram-positive bacteria differs from the bc_1 complexes in that cytochrome *b* is an $\alpha\beta$ dimer (31), with the two heme groups located in the larger, α subunit, whereas cytochrome b is a single polypeptide in all bc_1 complexes thus far characterized. The cytochrome *bf* complex also contains cytochrome *f* instead of cytochrome c_1. The primary difference between cytochrome c_1 and cytochrome *f* is that the axial ligands to the *c*-type heme are histidine and methionine and those to the *f*-type heme are histidine and lysine (125, 139).

To date no three-subunit *bf* complex has been isolated; in other words, no complex is known in which cytochrome *f* is associated with a cytochrome *b* which is a single subunit. Conversely, cytochrome c_1 has not been found in a complex in which cytochrome *b* is split into two subunits.

Cytochrome *b* is a hydrophobic, integral membrane protein, which varies in size from 38 to 48 kilodaltons (kDa) in species which contain bc_1 complexes. The α and β subunits of the cytochrome *b* of the *bf* complex have an additive mass comparable to that of the smaller of the single subunit *b*'s. The predicted secondary structure of yeast mitochondrial cytochrome *b* (Fig. 3) is exemplary of the cytochrome *b* of both mitochondrial and bacterial bc_1 complexes. The protein consists of nine helices, eight of which are predicted to be sufficiently hydrophobic to span the inner mitochondrial or bacterial plasma membrane. Labeling studies with reagents which probe the accessibility of the protein at the two membrane surfaces and the penetration of the protein within the hydrophobic membrane interior confirm that cytochrome *b* spans the membrane (8, 63). Helix 4 is less hydrophobic and is predicted to reside on the outer, P side of the membrane. This topographic arrangement of the helices is supported by the location of amino acids, which are the mutant loci conferring resistance to inhibitors that act specifically at center N or center P. These are discussed in the context of the proton motive Q cycle below. The *trans* membrane arrangement of eight of the helices and the peripheral location of helix 4 at the P side of the membrane are also supported by alkaline phosphatase-cytochrome *b* gene fusions in *R. sphaeroides*, which show that the phosphatase is directed to the periplasm as predicted by appropriately truncated forms of the cytochrome (R. Gennis, personal communication).

Cytochrome *b* contains two noncovalently bound heme groups. The two heme groups of cytochrome *b* are chemically identical, and EPR and magnetic circular dichroism spectra indicate that both heme groups are liganded to the protein through bis-histidine residues (28). Sequence comparisons of the cytochrome *b*'s from approximately 25 species (69) confirm that four histidines are conserved. One of the hemes (*b*-566) is thought to be liganded between the imidazoles of histidine 82 and histidine 183 (Fig. 3). The other heme (*b*-560) is expected to be liganded between histidine 96 and histidine 197. Both hemes are suspended between helices 2 and 5, which are thus expected to be closely juxtaposed.

Molecular models of helices 2 and 5 indicate that one or both of these helices must be distorted from a simple parallel juxtaposition to position the coordinating pairs of histidines proximal to and opposite each other (166). If the conserved proline 187 is in the *cis* conformation predicted from the molecular models, it introduces a discontinuity into helix 5 such that the histidines are not properly positioned for coordinating the hemes. This has led to the prediction that helices 2 and 5 are tilted approximately 20° with respect to each other (98). It is possible that helices 1, 2, 3, and 5 are arranged in a quartet motif with a left-handed twist and fourfold symmetry around their central axis, analogous to the α-helical bundle proposed by Link et al. (98).

The two heme groups of cytochrome *b* are electronically connected, traversing the mitochondrial or bacterial membrane, as evidenced by the fact that an applied membrane

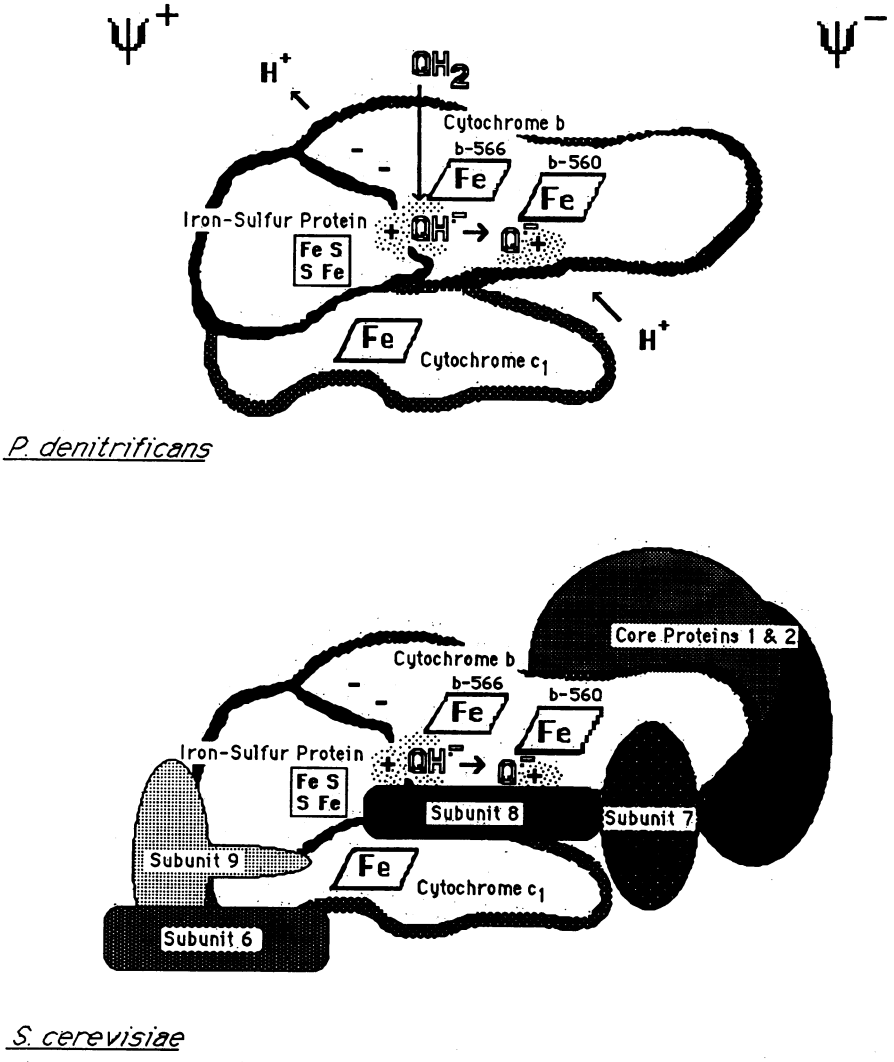

FIG. 2. Topographical arrangement of subunits in procaryotic and eucaryotic cytochrome bc_1 complexes. The top diagram depicts a postulated arrangement of the three redox group-containing subunits of the cytochrome bc_1 complex in the plasma membrane of bacteria such as *P. denitrificans*. The complex is asymmetric and spans the membrane, with the iron-sulfur protein and cytochrome c_1 located on the electropositive, periplasmic side of the membrane. Also depicted are the ubiquinol oxidase site (Q_p) at the iron-sulfur protein-cytochrome *b* interface, the ubisemiquinone/ubiquinone reductase site (Q_n) proximal to the *b*-560 heme group on cytochrome *b*, and proton uptake and release at these sites. The bottom diagram depicts the six supernumerary polypeptides of the *S. cerevisiae* mitochondrial cytochrome bc_1 complex superimposed on the three redox subunits.

potential will move an electron from one heme to the other under conditions where inhibitors are used to block electron transfer to or from cytochrome *b* and the iron-sulfur protein or cytochrome c_1 (172). In mitochondria the heme of *b*-560 appears to be near the center of the membrane bilayer, whereas that of *b*-566 is near the cytoplasmic, P surface (113). These positions relative to the two membrane surfaces are not obvious from the predicted disposition of the liganding histidines within the membrane-spanning helices (Fig. 3). Rather, the predicted secondary structure suggests that the heme of *b*-560 is near the N surface of the membrane, whereas that of *b*-566 is more internal. Similar topographical studies have not been conducted on the bacterial bc_1 complexes, but they should be, since the numerous additional proteins associated with the mitochondrial bc_1 complexes (see below) may block the accessibility of impermeable probes to the redox proteins of the complex and thus obscure the nature of their topographical disposition.

Cytochrome c_1 is an acidic protein, anchored to the P side of the mitochondrial or bacterial membrane by a hydrophobic domain at the C terminus. The predicted secondary structure of *S. cerevisiae* cytochrome c_1 is shown in Fig. 4. The *c*-type heme is covalently attached to the protein through two cysteine residues, and histidine and methionine residues are axial ligands to the heme. One of the unusual features of the predicted secondary structure of cytochrome c_1 is the presence of a series of hydrophobically located, β-pleated sheets.

The Rieske iron-sulfur protein is anchored to the mitochondrial or bacterial membrane by a stretch of hydrophobic amino acids at the N terminus. The predicted secondary structure of the *S. cerevisiae* iron-sulfur protein is shown in Fig. 5. The iron-sulfur cluster is coordinately liganded to the protein through two cysteines and two histidines and enveloped in a hydrophobic crevice, which appears to be shielded by two hydrophobic and two hydrophilic α-helices. The

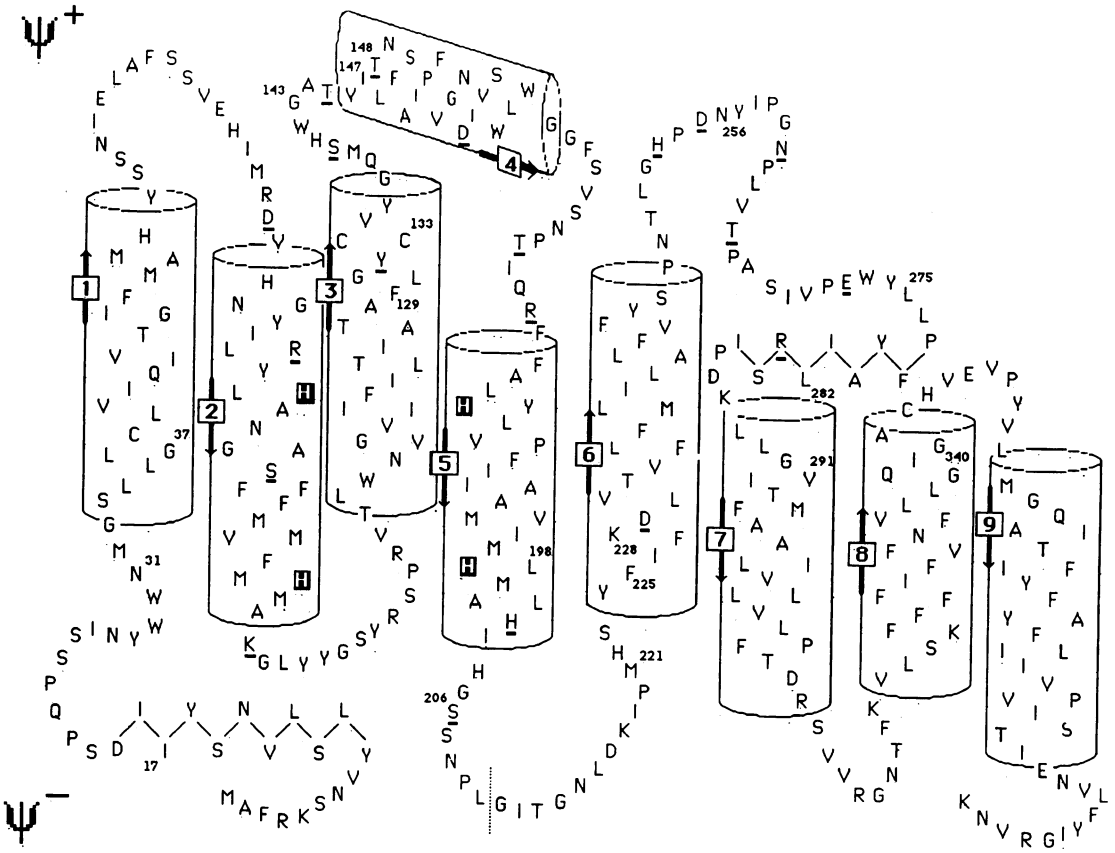

FIG. 3. Predicted secondary structure of *S. cerevisiae* cytochrome *b*. The predicted structure shows nine α-helices, numbered in open boxes, and two β-pleated sheets. Eight of the helices are predicted to be membrane spanning, while helix 4 is predicted to be located on the P side of the inner mitochondrial membrane. The dashed line in the loop between helices 5 and 6 shows the approximate point at which cytochrome *b* is split into α and β subunits in the cytochrome *bf* complexes. The four conserved histidines, thought to be the ligands to the two heme groups, are shadowed in black. The heme of *b*-566 is predicted to be coordinated to histidines 82 and 183, and that of *b*-560 is predicted to be coordinated to histidines 96 and 197. Amino acid residues which are loci of mutations conferring resistance to inhibitors which bind to cytochrome *b* are numbered (Table 3). Conserved protonic amino acids, considered to be possible proton-conducting residues, are underlined (Table 4). This structure is similar to those first proposed by Crofts et al. (33) and Brasseur (24).

protein is anchored to the bc_1 complex by its N terminus, so that trypsin liberates the majority of the mass of the protein as a single, prosthetic group containing water-soluble fragment (97).

The amino terminus of the mature yeast iron-sulfur protein begins at lysine 31 and is relatively enriched in polar amino acids (10). There is a stretch of hydrophobic amino acids distal to the N terminus, beginning at alanine 55; however, I do not think that these anchor the protein by spanning the membrane bilayer. Rather, it seems more likely that this hydrophobic sequence, and the polar amino acids in the N terminus, anchor the iron-sulfur protein to the complex by intercalation with cytochrome *b*. This would account for the fact that the iron-sulfur protein is specifically dissociated from the bc_1 complex by low concentrations of cholate in the presence of guanidine (150) or when mitochondria are washed with sodium carbonate (66).

The three redox prosthetic group-containing subunits of the *P. denitrificans* bc_1 complex resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Fig. 6. This figure also illustrates a potentially useful feature of the bacterial bc_1 complexes, in that cytochrome *b* can be separated from cytochrome c_1 and the Rieske iron-sulfur protein quantitatively in a single step (119). This single-step purification works equally well with the cytochrome *b* of *R. sphaeroides* (119) and presumably can be applied to the complexes from *Rhodospirillum rubrum* and *R. capsulatus*.

The single-step separation of cytochrome *b* takes advantage of the fact that Triton X-114 undergoes a microscopic phase separation at 20°C. Above this temperature, cytochrome *b* partitions exclusively into the detergent phase, whereas cytochrome c_1, the Rieske iron-sulfur protein, and the fourth subunit of the *R. sphaeroides* complex (see below) are retained in the aqueous phase and can be further resolved by gel filtration chromatography. This simple, one-step purification of cytochrome *b* should be especially useful for isolating cytochrome *b* before peptide mapping, after affinity labeling of amino acid residues involved in proton conduction or ubisemiquinone stabilization.

The hemes of cytochromes *b* and c_1 are red and can be resolved optically by differential reduction of the cytochrome bc_1 complex (Fig. 7). If the bc_1 complex is reduced in a stepwise fashion, progressing from a relatively high reduction potential to lower potentials, cytochrome c_1 is reduced first (Fig. 7b). As the reduction potential is lowered, the high-potential portion of cytochrome *b*, having an absorption maximum at approximately 560 nm, is reduced next, along with the cytochrome c_1 (Fig. 7c and d). If the absorption due to cytochrome c_1 is subtracted from these

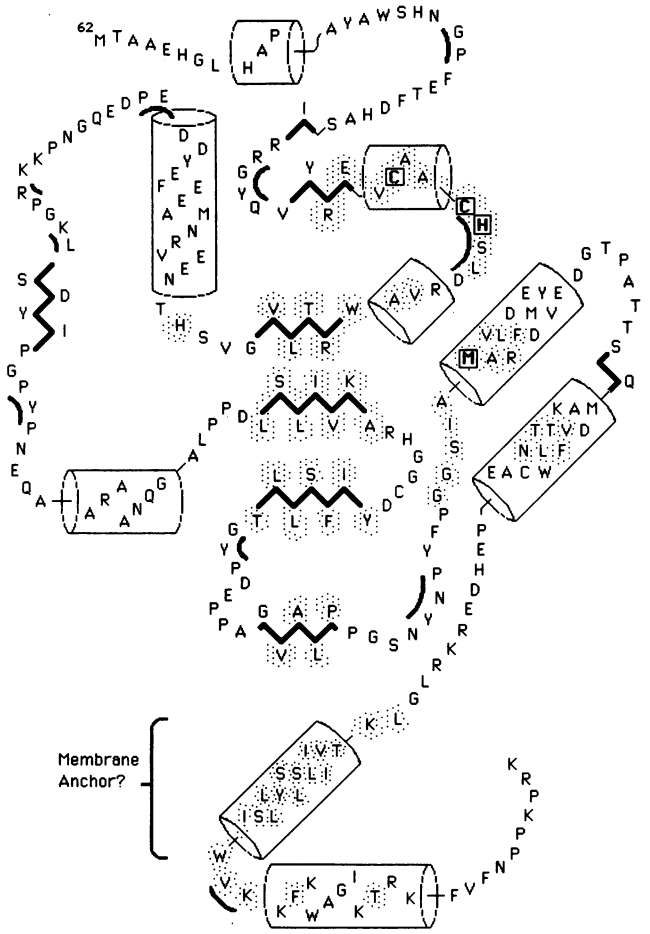

FIG. 4. Predicted secondary structure of *S. cerevisiae* cytochrome c_1. Methionine 62 is the N terminus of the mature protein. The figure shows predicted regions of α-helix, β-pleated sheet, and β-turns. Amino acids in regions predicted as hydrophobic are enclosed by a stippled area. The two cysteines to which the heme is covalently attached and the conserved histidine and methionine, which are the fifth and sixth axial ligands to the heme, are shown in bold type, enclosed in boxes. The postulated C-terminal hydrophobic membrane anchor is depicted as an α-helix, presumably capable of spanning the membrane, although the predicted secondary structure of this region includes a β-pleated sheet.

composite spectra, the spectrum of the high-potential cytochrome *b*-560 is resolved as a single, nearly symmetrical absorption peak at 559 to 560 nm (Fig. 7f and g).

When the high-potential *b*-560 is completely reduced with menaquinol, a portion of the low-potential *b*-566 is also reduced and appears as a long-wavelength shoulder in a difference spectrum of the cytochrome *b* reduced with menaquinol minus that reduced with ubiquinol (Fig. 7h). An absorption spectrum consisting of only the low-potential *b*-566 is obtained by reducing the complex with dithionite and subtracting the spectrum of the complex reduced with menaquinol (Fig. 7i).

Although the two *b* heme groups are chemically identical, they manifest markedly different optical properties and oxidation-reduction potentials (Fig. 7). In *P. denitrificans* the high-potential *b*-560 titrates as two components, having midpoint potentials of +30 and +120 mV (E. A. Berry and B. L. Trumpower, unpublished results). This biphasic potentiometric behavior of the single *b*-560 heme group has been observed in the bc_1 complexes of numerous species (39) and is due to association of ubiquinone at a site proximal to *b*-560 and its reduction to ubiquinol (128).

The low-potential *b*-566 titrates as a single component, having a midpoint potential of −95 mV in *P. denitrificans*. The low-potential heme has a split α-band, with a low-wavelength shoulder at approximately 558 nm. This splitting of the absorption band reflects steric strain on the low-potential heme group and may result from a torsional twist in helix 5, as discussed above.

In *Rhodospirillum rubrum*, cytochromes *b*-560 and *b*-566 titrate with midpoint potentials of −33 and −90 mV, respectively (85), whereas in *R. sphaeroides* the two cytochrome *b* hemes exhibit midpoint potentials of +50 and −80 mV (106). These differences in midpoint potentials of the two *b* hemes, which have been observed in all cytochrome bc_1 complexes thus far examined, derive from differences in the dielectric and charge environments created by amino acid residues around the redox centers and the geometry of the axial histidine ligands to the hemes. The amino acid residues that contribute to these differences are not known at present.

Cytochrome c_1 and the Rieske iron-sulfur protein have midpoint potentials of +190 and +265 mV, respectively, in *P. denitrificans* (Berry and Trumpower, unpublished). A similar increment in potentials, with the iron-sulfur cluster being more readily reducible than the c_1 heme, occurs in all species for which these two redox components have been compared (148). This difference in midpoint potentials of the iron-sulfur protein and cytochrome c_1, along with the topographical disposition of the iron-sulfur cluster in a more electropositive location than the heme of cytochrome c_1, in relation to the membrane potential, may contribute to the retarding effect of the proton motive force on electron transfer through the bc_1 complex (148).

The 2Fe-2S cluster of the Rieske iron-sulfur protein contains an unpaired electron when the cluster is reduced and thus gives rise to an EPR signal. The spectrum resulting from the Rieske cluster in *P. denitrificans* is shown in Fig. 8. This iron-sulfur cluster is atypical of binuclear clusters in that g_{av} is approximately 1.90, whereas for most ferredoxins g_{av} is in the range 1.94 to 1.98. The midpoint potential of the Rieske iron-sulfur cluster is also 400 to 600 mV more positive than those of ferredoxins. Recent evidence indicates that the ligands to the Rieske iron-sulfur cluster are two cysteines and two histidines (62, 91, 144), as opposed to four cysteine ligands in ferredoxins. Since histidine is less strongly electron donating than sulfur, this difference in ligands accounts for the high midpoint potential of the Rieske cluster and may also account for the atypical *g* value. That one or more of the ligands to this iron-sulfur cluster must be less electron donating than sulfur was predicted on theoretical grounds by Blumberg and Peisach in 1974 (16).

The Rieske iron-sulfur protein is the oxidant for ubiquinol in the proton motive Q cycle pathway of electrons through the bc_1 complex, and the line shape of the EPR signal arising from the iron-sulfur cluster is influenced by the redox state of ubiquinone. In addition, both the line shape and the midpoint potential of the cluster are affected by structural analogs of ubiquinone which inhibit respiration. When the oxidation-reduction potential is increased from −100 to +140 mV, there is a sharpening and shift of all three *g* values (Fig. 8) (107). This change in the EPR signal correlates with oxidation of ubiquinol to ubiquinone, as was first demonstrated with the bc_1 complex from *S. cerevisiae* (138) and subsequently with that from *R. sphaeroides* (104).

Hydroxy analogs of ubiquinone, such as 5-*n*-undecyl-

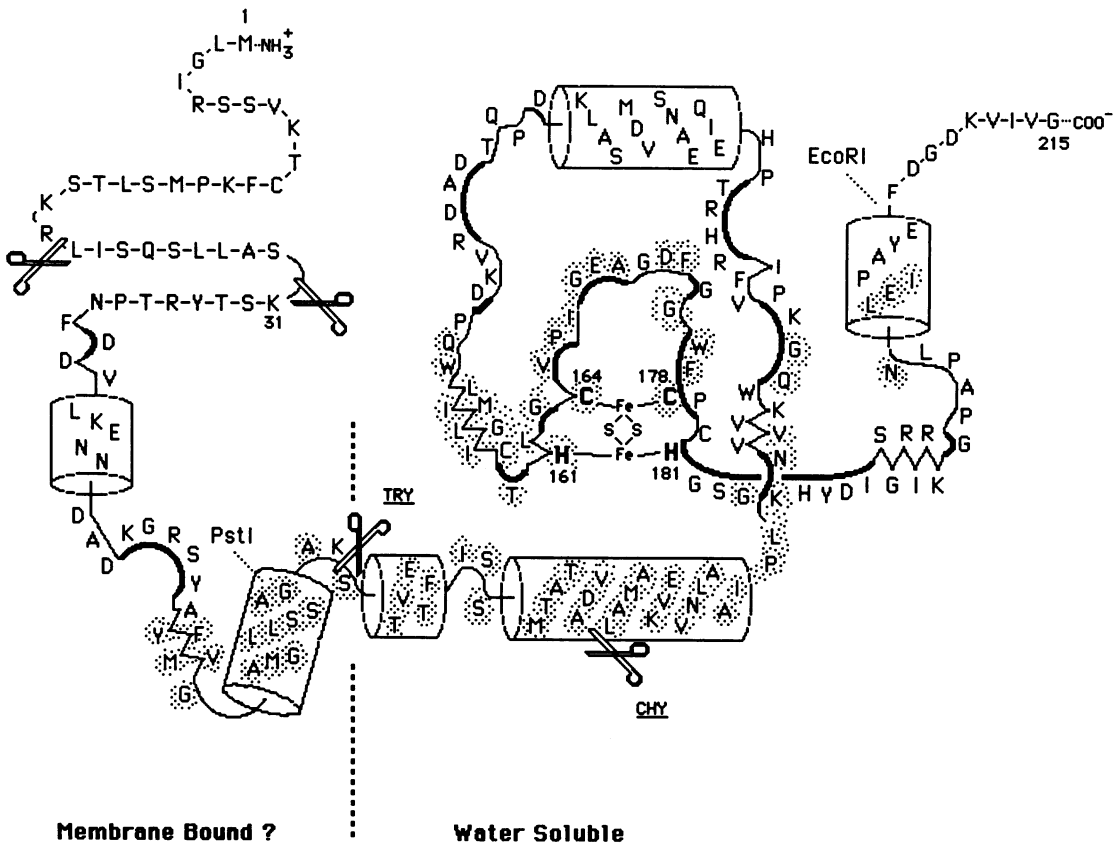

FIG. 5. Predicted secondary structure of *S. cerevisiae* Rieske iron-sulfur protein. Lysine 31 is the N terminus of the mature protein. The figure shows predicted regions of α-helix, β-pleated sheet, and β-turns. Amino acids in regions predicted as hydrophobic are enclosed by a stippled area. The 2Fe-2S cluster is shown liganded through two conserved cysteines (Cys-164 and Cys-178) and two conserved histidines (His-161 and His-181). Cysteine 159 and cysteine 180 are also conserved in all Rieske iron-sulfur proteins sequenced to date (10). Also shown are the sites at which trypsin (TRY) and chymotrypsin (CHY) cleave the membrane-bound iron-sulfur protein to release a water-soluble portion of the protein and the sites in the N terminus where the protein is posttranslationally processed in two proteolytic steps during import into the mitochondria. The regions of the protein defined by *Pst*I and *Eco*RI sites in the *RIP1* gene, which were used to mutagenize the cloned gene (11, 100), are also shown. (Reproduced, with minor modifications, from reference 11 with permission.)

6-hydroxy-4,7-dioxobenzoxythiazole (UHDBT), inhibit electron transfer through the cytochrome bc_1 complex (152), and binding of UHDBT is proportional to the amount of iron-sulfur protein in the complex (20). Bowyer et al. (19) showed that UHDBT increases the midpoint potential of the Rieske iron-sulfur protein in *R. capsulatus* and causes a broadening of the EPR spectrum. The increase in midpoint potential indicates that UHDBT binds to the bc_1 complex approximately 15-fold more tightly when the iron-sulfur cluster is reduced. The effect of UHDBT on the line shape of the EPR spectrum has also been observed in mitochondria (20) and is similar but not identical to the change resulting from reduction of ubiquinone to ubiquinol (107). My interpretation of the effects of UHDBT and myxothiazol (see below) is that the ubiquinol oxidase site is composed of structural domains from both the Rieske iron-sulfur protein and cytochrome *b* and that UHDBT and center P inhibitors such as myxothiazol bind in an overlapping manner at this site, which is otherwise occupied by ubiquinone when the iron-sulfur cluster is reduced. UHDBT and center P inhibitors prevent the product of the ubiquinol oxidase reaction from occupying its site and thus block ubiquinol oxidase activity of the complex.

In bacteria having branched electron transfer chains and multiple pathways of ubiquinol oxidation, UHDBT may have multiple sites of inhibition, owing to its structural similarity to ubiquinol. Kucera et al. (88) found that the alternate oxidase pathway in *P. denitrificans* is inhibited by UHDBT at concentrations 1 to 2 orders of magnitude lower than those which inhibit the bc_1 complex.

In addition to the protein-bound redox prosthetic groups, the cytochrome bc_1 complex contains a site which stabilizes ubisemiquinone by approximately 10 orders of magnitude. This semiquinone is sufficiently stable that an EPR signal arising from the semiquinone radical can be observed when the bc_1 complex is poised at a potential near the midpoint potential of the ubiquinone-ubiquinol couple. The ubisemiquinone radical signal observed in *P. denitrificans* is shown in Fig. 9. Under these conditions the concentration of ubisemiquinone reached a maximum of 0.57 molecule per complex during the potentiometric titration. Using a value of approximately 3 molecules of ubiquinone associated with the purified *P. denitrificans* bc_1 complex (180), one can calculate that this corresponds to a stability constant of 0.22 for the ubisemiquinone in the three-subunit complex. The stability constant of ubisemiquinone in a hydrophobic environment, in the absence of any stabilization by protein, is estimated to be 10^{-10} (110).

The site which stabilizes ubisemiquinone is center N, in the terminology of the proton motive Q cycle (see below).

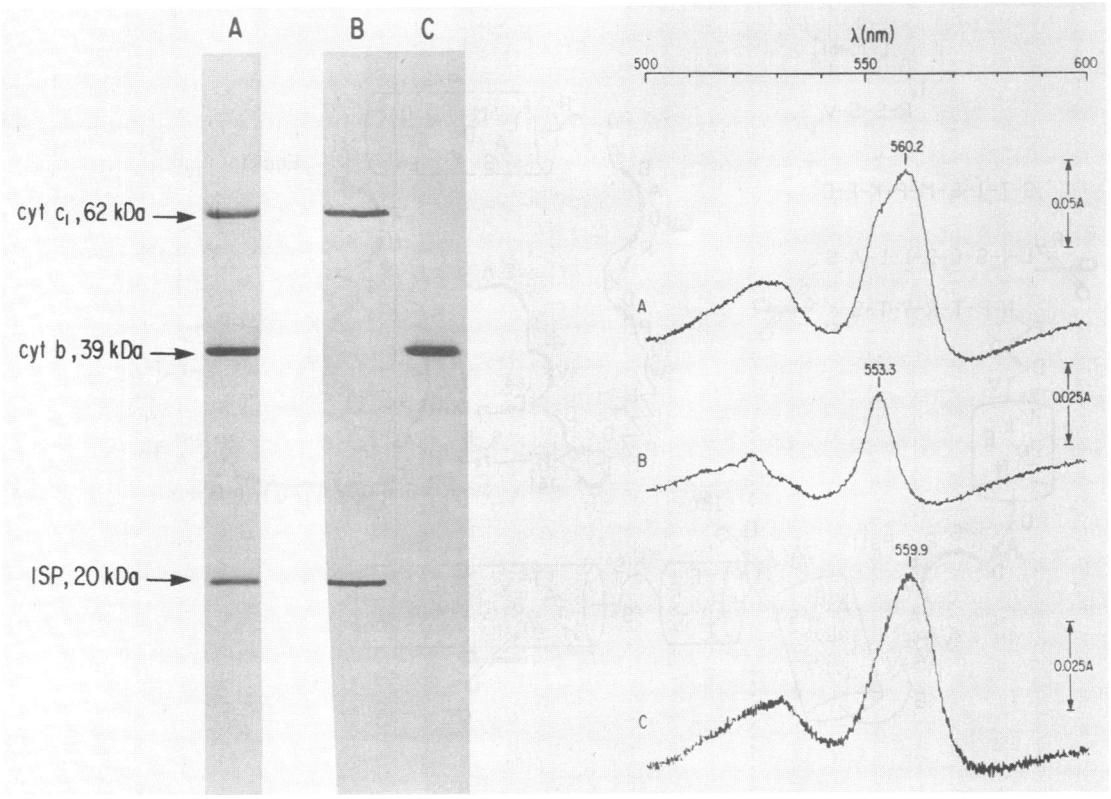

FIG. 6. Separation of the three subunits of the *P. denitrificans* bc_1 complex by SDS-PAGE and single-step purification of cytochrome *b*. (A) Gel showing the three-subunit complex purified from *P. denitrificans* (180). (B) Gel showing cytochrome c_1 and the Rieske iron-sulfur protein subunits. (C) Gel showing cytochrome *b* after single-step purification of cytochrome *b* by microscopic phase separation into Triton X-114 (119). The tracings to the right show the optical absorption spectra of the three-subunit bc_1 complex (A), the cytochrome c_1 and iron-sulfur protein fraction (B), and the cytochrome *b* fraction (C) after phase separation of the subunits by Triton X-114. (Reproduced from reference 17, with permission.)

This ubiquinone/ubisemiquinone reductase site is located on cytochrome *b*, near the *b*-560 heme group (Fig. 2) to which it is diamagnetically exchange coupled (38), and is identical to or proximal to the antimycin-binding site (Fig. 3). The ubisemiquinone radical is displaced from its binding site, or the conformation of the site is altered by binding of antimycin such that it does not stabilize the semiquinone. Consequently, the EPR signal arising from the stable ubisemiquinone is eliminated coincident with binding of the inhibitor.

It is uncertain whether a distinct Q-binding protein, in addition to cytochrome *b*, is also required for stabilizing ubisemiquinone in eucaryotic bc_1 complexes. On the basis of affinity labeling with ubiquinone analogs, Yu et al. (185) suggested that the 14-kDa subunit 7 of the yeast complex is a Q-binding protein. The 14-kDa subunit 7 of the yeast complex is not homologous to the 9.5-kDa subunit 7 of the bovine mitochondrial bc_1 complex, which is similarly labeled by azido derivatives of ubiquinone (184). Although one or more nonredox subunits may be part of, or physically proximal to, the Q_n^- site in mitochondria (Fig. 2), stabilization of ubisemiquinone clearly does not require any Q-binding protein in *P. denitrificans*, since the stable semiquinone radical is observed in the three-subunit complex (107).

In my view, it is also unlikely that a distinct Q-binding protein is required for ubisemiquinone stabilization in either *R. sphaeroides* or *R. capsulatus*, which are phylogenetically very similar to *P. denitrificans*, even though these bc_1 complexes contain a fourth polypeptide which is labeled by an azido derivative of ubiquinone (186) (see below). Whether the three-subunit bc_1 complex of *Rhodospirillum rubrum* exhibits a similar stable semiquinone radical has not been reported.

Contrary to a common misconception, there is no high-affinity binding site for ubiquinone or ubiquinol in the bc_1 complex, even though the complex stabilizes ubisemiquinone by 10 orders of magnitude. The partition coefficient for ubiquinone between Triton X-100 and the bc_1 complex of *N. crassa* in the protein-detergent mixed micelle is close to unity (171), and the existence of a ubisemiquinone radical signal does not require that there is a high-affinity binding site for ubiquinone or ubiquinol in the bc_1 complex. Rather, the observed stability constants of ubisemiquinone (107, 114) reflect the fact that the relative affinities of the Q_n^- site for the three redox forms, ubiquinone, ubisemiquinone, and ubiquinol, are approximately equal.

Proteins Lacking Redox Prosthetic Groups

The bc_1 complexes purified from both *R. sphaeroides* and *R. capsulatus* differ from those of *P. denitrificans* and *Rhodospirillum rubrum* in that they contain a fourth polypeptide, lacking a redox prosthetic group, as seen in the electrophoresis gels in Fig. 10. It is not known whether this fourth polypeptide is analogous in these two bacteria, whether they are bona fide subunits of the complexes, or whether they have sequence homology to any of the super-

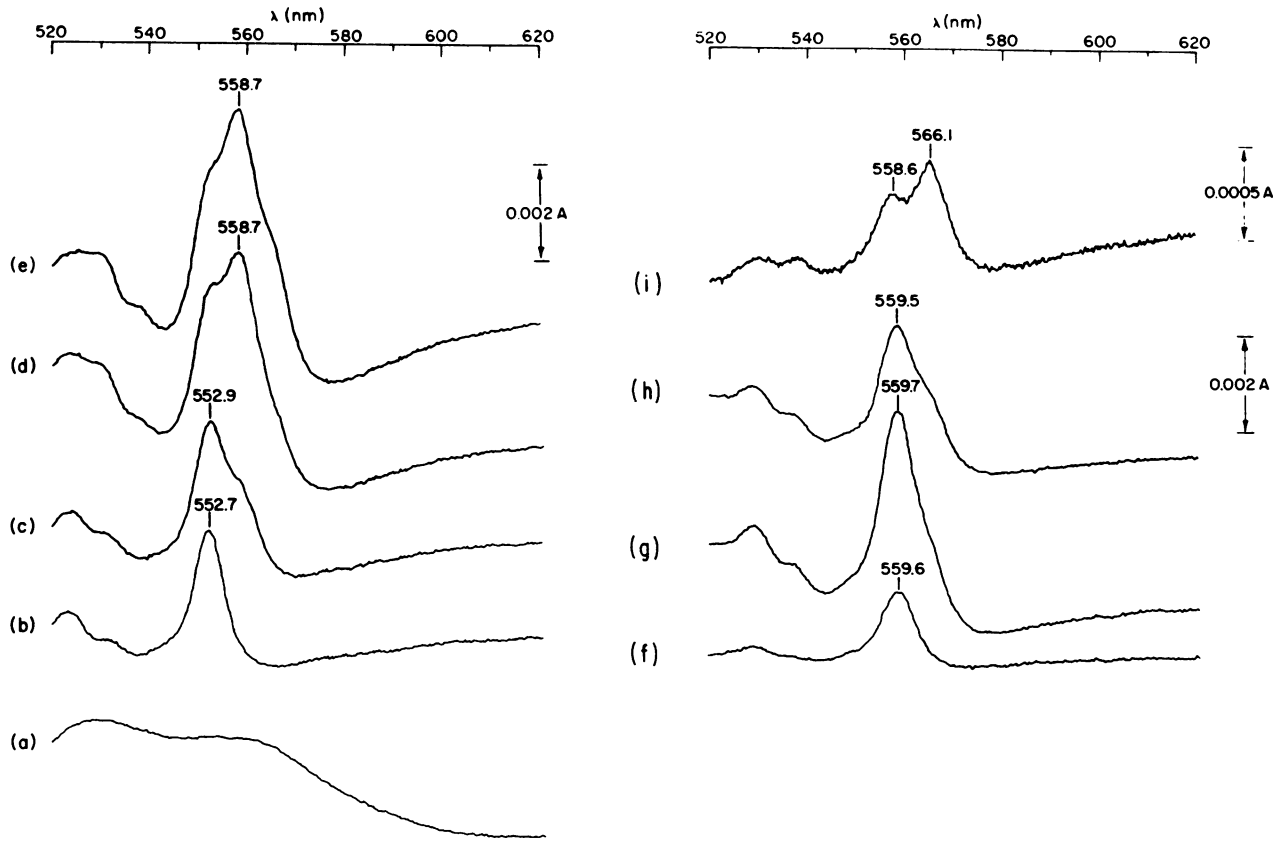

FIG. 7. Optical spectra of *P. denitrificans* bc_1 redox components. The tracings show absorption difference spectra of the cytochromes *b* and c_1 of the purified cytochrome bc_1 complex. Spectrum (a) is of the oxidized complex. This spectrum was stored on a digital oscilloscope and then used as a base line, which was subtracted to obtain spectra (b) through (e). Spectrum (b) is of cytochrome c_1, which was obtained as a difference spectrum of the ascorbate-reduced complex minus that of the oxidized complex. Spectrum (c) is of cytochrome c_1 plus the high-potential portion of cytochrome *b*, which was obtained as a difference spectrum of the ubiquinol-reduced complex minus that of the oxidized complex. Spectrum (d) is of cytochrome c_1 plus the high-potential portion and most of the low-potential portion of cytochrome *b*; it was obtained as a difference spectrum of the menaquinol-reduced complex minus that of the oxidized complex. Spectrum (e) is of cytochrome c_1 plus the high-potential portion and all of the low-potential portion of cytochrome *b*; it was obtained as a difference spectrum of the dithionite-reduced complex minus that of the oxidized complex. Spectrum (f) is of the high-potential portion of cytochrome *b*, which was obtained by subtracting spectrum (b) from spectrum (c). Spectrum (g) is of the high-potential cytochrome *b* and a portion of the low-potential cytochrome *b*, obtained by subtracting spectrum (b) from spectrum (d). Spectrum (h) is of a portion of the low-potential cytochrome *b*, obtained by subtracting spectrum (c) from spectrum (d). Spectrum (i) is of the lowest-potential portion of cytochrome *b*, obtained by subtracting spectrum (d) from spectrum (e).

numerary polypeptides of the mitochondrial complexes. The possibility also exists, and merits exploration, that the bc_1 complexes of *P. denitrificans* and *Rhodospirillum rubrum* contain additional subunits in situ analogous to those in *R. sphaeroides* and *R. capsulatus*, but that these subunits are lost during purification of the complexes from the former two bacteria.

One of the most intriguing differences between the bc_1 complexes of mitochondria and those of bacteria is that the former contain six or more polypeptides which lack redox prosthetic groups, in addition to the three redox group-containing polypeptides which are common to both procaryotes and eucaryotes. I have referred to these as supernumerary polypeptides to emphasize that they are in excess of the number of polypeptides needed for the electron transfer and energy-transducing activities of the bc_1 complex, as evidenced by the fact that the three-subunit complex from *P. denitrificans* has the same electron transfer and proton-translocating activities as the bc_1 complexes which contain these extra subunits (181). Although the subunit compositions of the *bf* complexes from algae and chloroplasts are less well established, it appears that the *bf* complexes of chloroplasts contain one or more supernumerary polypeptides, in addition to the redox group-containing subunits (65), but that the former readily dissociate from the latter during purification (68).

A comparison of the subunit compositions of the complexes from *P. denitrificans* and *S. cerevisiae* is shown in Fig. 11. The three subunits of the bacterial complex are the three redox proteins cytochrome *b*, cytochrome c_1, and Rieske iron-sulfur protein. In addition to the three prosthetic group-containing proteins, the yeast complex contains six proteins which have no counterparts in the bacterial complex. The bc_1 complex from *N. crassa* resembles that from *S. cerevisiae* in that it, too, contains six supernumerary polypeptides in addition to the three redox proteins (80, 143).

All six of the supernumerary polypeptides of the yeast bc_1 complex are bona fide subunits, and not impurities, since removal of any one of them by deleting their genes has an effect on the assembly, stability, or catalytic activity of the

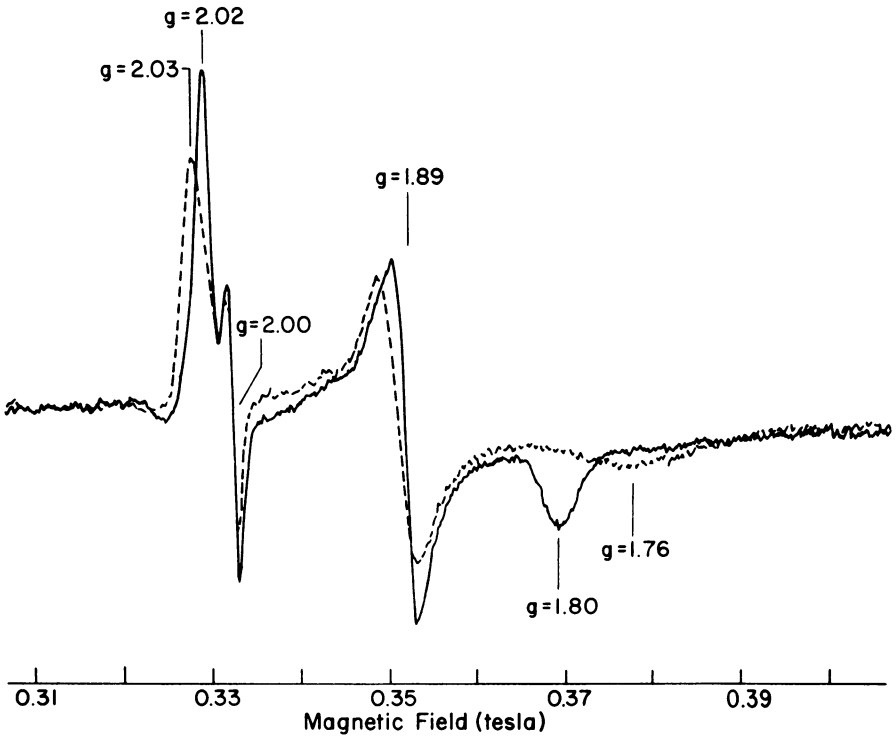

FIG. 8. EPR spectra of the Rieske iron-sulfur cluster in the purified three-subunit cytochrome bc_1 complex of *P. denitrificans* (107). The spectrum shown by the dashed line was obtained at an applied redox potential of −100 mV; that shown by the solid line was obtained at a potential of +140 mV. The cytochrome bc_1 complex was suspended at 21 μM in 50 mM potassium phosphate–350 mM KCl–0.2 g of dodecyl maltoside (pH 8.5) per liter. Spectra were obtained in collaboration with Steve Meinhardt and Tomoko Ohnishi at the University of Pennsylvania (107).

complex. Several considerations suggest that these six polypeptides are peripheral to and envelope the three redox proteins. Hydropathy calculations, using the amino acid sequences deduced from the sequenced genes, indicate that the supernumerary polypeptides are largely hydrophilic. It therefore seems that some of these subunits are held to the complex through interactions with other proteins in the complex and do not rely on the low dielectric constant of the membrane as an enthalphy source.

The subunits lacking redox prosthetic groups are located asymmetrically in the bc_1 complex (Fig. 2, bottom). The topography of these subunits and their proximity to one or more of the redox subunits have been deduced by chemical cross-linking, covalent modification with impermeable reagents, distribution of subunits during subfractionation of the complex, accessibility to antibodies and proteases, and electron-microscopic image reconstruction. The arrangement of subunits presented in Fig. 2 incorporates information from topography studies on the bc_1 complexes of *S. cerevisiae*, *N. crassa*, and beef heart mitochondria. These studies have been reviewed and extended by Gonzalez-Halphen et al. (59) and will not be detailed here.

Core proteins 1 and 2 (Fig. 2) are proximal to each other (59), lack any obvious membrane-anchoring domains (116, 155), and extend into the matrix space of the mitochondria (80). Core protein 1 of the yeast complex migrates on SDS-PAGE with an apparent molecular mass of 44 kDa (Fig. 11), but from the sequence of the cloned gene (155) the mature protein has an approximate molecular mass of 49.7 kDa, assuming that the presequence which is posttranslationally removed has a molecular mass of 0.5 kDa (158). Core protein 2 migrates on SDS-PAGE with an apparent molecular mass of 40 kDa (Fig. 11). The sequence of the gene and the N-terminal amino acid sequence of the mature protein indicate that the actual molecular mass is 38.7 kDa (116).

If core protein 1 is genetically deleted from *S. cerevisiae*, heme is not inserted into apocytochrome *b*, and the assembly of the bc_1 complex is blocked at an early step (155). If core protein 2 is deleted from *S. cerevisiae*, heme-dependent maturation of cytochrome *b* is also impaired, although only partially. The resulting yeast strain retains approximately 5% of the wild-type bc_1 complex activity and grows slowly on nonfermentable carbon sources (116). These findings suggest that, at least in *S. cerevisiae*, addition of the hemes to cytochrome *b* is contingent on a specific folding or conformation of apocytochrome *b* which requires the presence of the two core proteins (155).

The two core proteins are rather loosely associated with the *N. crassa* bc_1 complex and dissociate together as a subcomplex when the complex is exposed to detergent and small amounts of salt. Core protein 1 serves two functions in *N. crassa*. Schulte et al. (134) discovered that this subunit of the *N. crassa* bc_1 complex is identical to the processing-enhancing protease (PEP) which stimulates the proteolytic activity of the matrix-processing protease (70). Core protein 1 purified from the bc_1 complex, or the intact complex, substituted for PEP in the protease-enhancing assay. This suggests that in *N. crassa*, the posttranslational proteolytic processing which occurs coincident with translocation of nucleus-encoded proteins across the inner mitochondrial membrane occurs on the bc_1 complex, at the matrix surface of the inner membrane.

Core protein 1 must also have some function intrinsic to

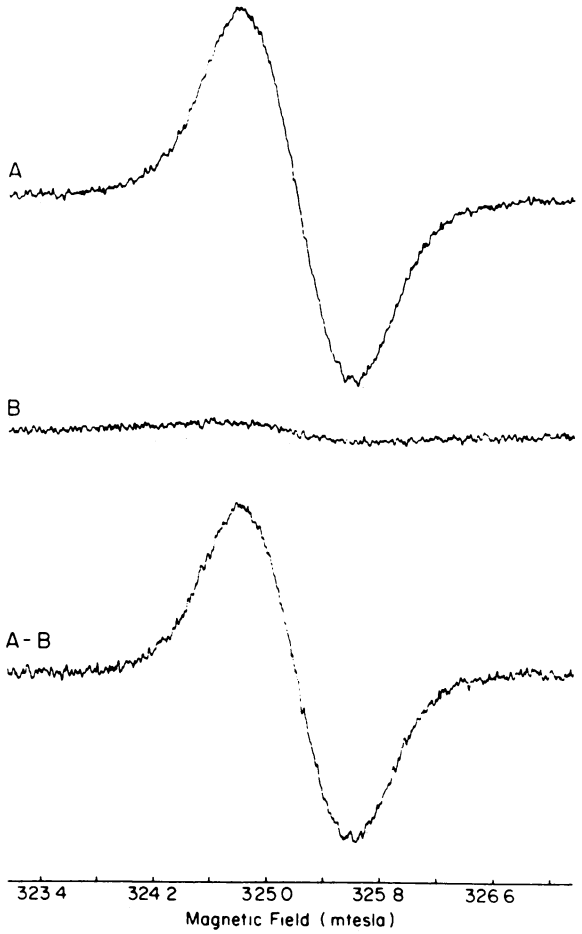

FIG. 9. EPR spectra of the antimycin-sensitive ubisemiquinone radical in the cytochrome bc_1 complex of *P. denitrificans* (107). Spectra A and B were obtained by suspending the complex in buffer at pH 8.5, as in Fig. 8, and posing the potential at approximately +50 mV. Spectrum B demonstrates the disappearance of the radical signal in the presence of antimycin. The bottom spectrum is a calculated difference spectrum of A minus B. (Reproduced from reference 107, with permission.)

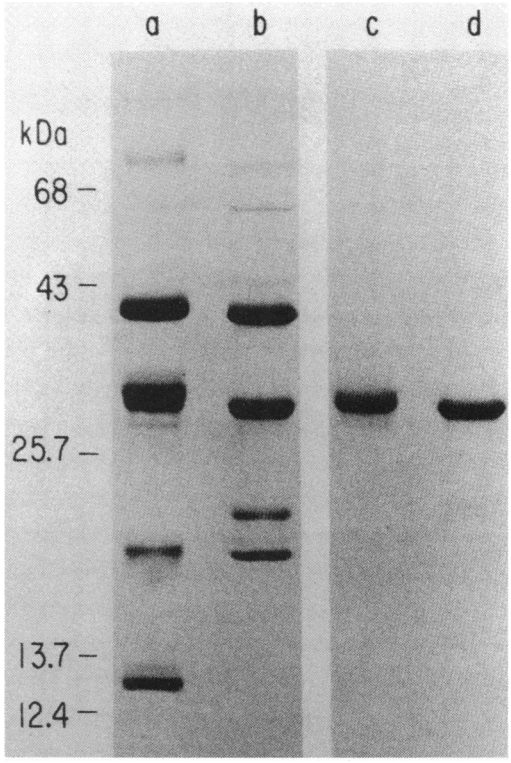

FIG. 10. SDS-PAGE of *R. sphaeroides* and *R. capsulatus* bc_1 complexes (99). Lanes a and b, stained with Coomassie blue, show the four subunits of the *R. sphaeroides* and *R. capsulatus* complexes, respectively. Lanes c and d show the same gel, stained for heme, which identifies cytochrome c_1 in the two complexes. The putative fourth, nonredox group-containing subunit is the fastest-migrating polypeptide in both of the complexes. Cytochrome *b* is the slowest-migrating polypeptide, and the iron-sulfur protein is the weakly staining polypeptide in both complexes. The numbers to the left designate the migration positions of molecular mass standards. (Reproduced from reference 99, with permission.)

the bc_1 complex, since core protein 1 of *S. cerevisiae* is different from the *MAS1* protein (176), the protease-enhancing protein which is homologous to *N. crassa* PEP (70, 134). *S. cerevisiae*, unlike *N. crassa*, has very low levels of cytochrome bc_1 complex when grown fermentatively, owing to catabolite repression. However, promitochondria of such *S. cerevisiae* cells still must import and process some nucleus-encoded proteins. It is thus not surprising that the constitutively required protease-enhancing protein and the repressible respiratory complex core protein 1 should be different gene products in this organism.

Although core proteins 2 of *S. cerevisiae* and *N. crassa* have no protease-enhancing activity, they do show a low degree of sequence similarity to core proteins 1 of *S. cerevisiae* and *N. crassa*, the *S. cerevisiae* MAS1 protein, and the *N. crassa* PEP. The four proteins thus constitute a family (134) and most probably have a common phylogenetic origin. The point during evolution at which they became structurally and functionally distinct, and whether they acquired a function simultaneously in mitochondrial protein import and respiration coincident with the appearance of the organelle in primitive eucaryotes are interesting, unanswered questions.

In my laboratory we have observed that core protein 2 consisently migrates as a doublet on SDS-PAGE (P. O. Ljungdahl and B. L. Trumpower, Biophys. Soc. J. **40**:196a, 1985) (Fig. 11). This microheterogeneity may be due to the presence of some incompletely processed core protein 2 precursor, in line with the observation that relatively high levels of the precursor of this subunit were observed in pulse-chase labeling experiments (158). If the purified bc_1 complex does consist of two populations, one containing mature core protein 2 and the other containing precursor core protein 2, it would be interesting to know whether both are catalytically active.

Subunit 6 of the yeast bc_1 complex migrates with an apparent molecular mass of 17 kDa on SDS-PAGE, but the molecular mass of the mature protein, deduced from the sequence of the cloned gene, is 14.5 kDa (156). This subunit is somewhat homologous to the hinge protein of the beef heart mitochondrial bc_1 complex and is thought to be located on the cytoplasmic surface of the inner membrane, where it facilitates cytochrome c_1 interaction with cytochrome *c* (82). Subunit 6 is highly acidic and contains an uninterrupted stretch of 24 acidic amino acids (156).

Subunit 6 also contains a domain which resembles one of

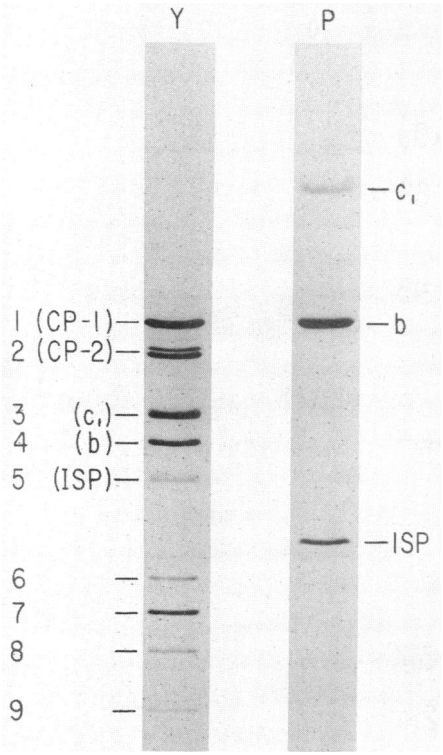

FIG. 11. SDS-PAGE of *P. denitrificans* and *S. cerevisiae* bc_1 complexes. The yeast complex consists of nine subunits, numbered to the left. Only three of the yeast subunits, cytochrome *b*, cytochrome c_1, and iron-sulfur protein, contain redox prosthetic groups. These three redox group-containing subunits are the only subunits present in the purified complex from *P. denitrificans*. The functions of the non-redox-group-containing subunits in the yeast complex are not known. Subunit 2 of the yeast complex, core protein 2, splits into two bands (Ljungdahl and Trumpower, abstract). The reason for this microheterogeneity is not known, but may be due to incomplete processing of the N-terminal presequence. The photograph is a composite of two different electrophoresis gels; consequently, the migration positions of subunits in the two complexes cannot be compared.

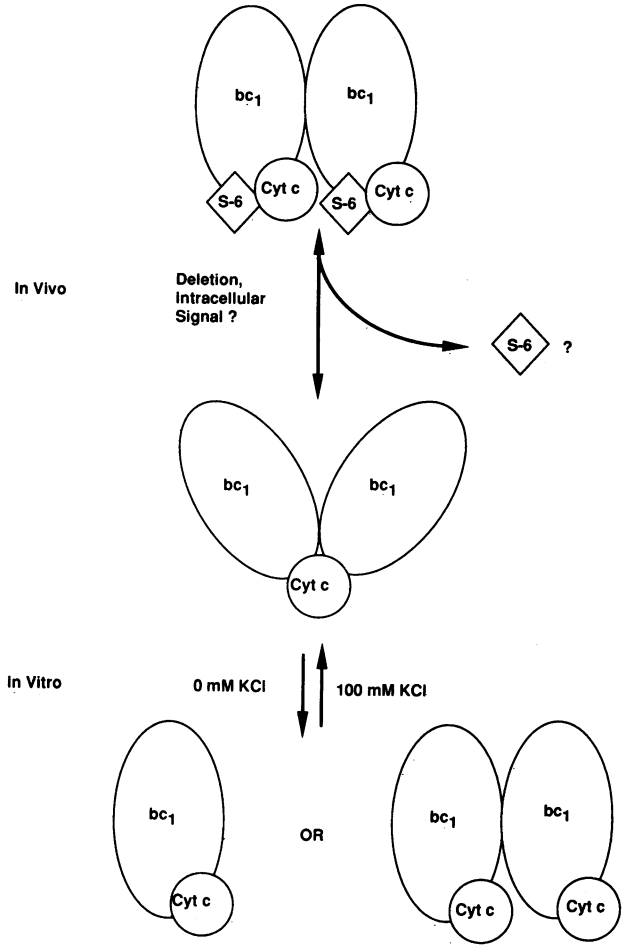

FIG. 12. Model for regulation of cytochrome *c* reductase activity of the dimeric bc_1 complexes of mitochondria. The model proposes that the proton motive force across the inner mitochondrial membrane mimics the genetic deletion of subunit 6 (S-6), causing a reversible dissociation or conformational change of the acidic subunit, thus halving the activity of the dimeric bc_1 complex. The artificial reversal of this drop in activity which is observed in vitro when the purified complex lacking subunit 6 is exposed to low ionic strength is achieved in vivo when the subunit reassociates with the complex.

the calcium-binding domains of yeast calmodulin (130). This homology spans 24 residues in the middle of the protein and includes 13 residues which are identical or highly conserved. This calmodulinlike motif is not present in the beef heart protein. The predicted secondary structure of subunit 6 indicates an unusually high degree of α-helix (85%) and a single hydrophobic hairpin at the C terminus, which might anchor the protein to the membrane. This protein thus has properties reminiscent of signal-transducing proteins, but to date there is no evidence for a calcium-modulated change in activity of the bc_1 complex.

Deletion of the gene encoding subunit 6 does not impair the growth of *S. cerevisiae* on nonfermentable carbon sources (133). Ubiquinol-cytochrome *c* reductase activity of mitochondria from the deletion strain was decreased 50% under conditions where the activity is zero order with respect to cytochrome *c*, and there was a similar decrease in the first-order rate constant for cytochrome *c* reduction (M. E. Schmitt and B. L. Trumpower, submitted for publication). Both the zero-order rate and the first-order rate constant for cytochrome *c* reduction could be recovered to those of the parental strain by conditions of low ionic strength that lead to a dimer-to-monomer transition in cytochrome *c* reductase (112). The zero-order rate and first-order rate constants for cytochrome *c* reduction in membranes from the parental wild-type yeast showed very little change coincident with this change in ionic strength.

The 50% drop in both V_{max} and k_1 for cytochrome *c* reductase activity indicates that half the cytochrome bc_1 complexes are inactive in the strain from which subunit 6 has been deleted, under conditions where the cytochrome bc_1 complex is thought to be dimeric (95). In formal kinetic terms, the cytochrome bc_1 complex exhibits half-of-the-sites reactivity when subunit 6 is deleted. We propose that the artificially imposed, genetic deletion of subunit 6 mimics a reversible dissociation or conformational change of this subunit in situ, resulting in silencing of the cytochrome *c* reduction site in one of the two monomers in the structurally dimeric bc_1 complex (Fig. 12). This mechanism for inactivating one of the monomers is consistent with the view that subunit 6 facilitates the formation of a complex between cytochrome c_1 and cytochrome *c* (82). For this model to

have physiological relevance, there must be some effector or intracellular signal which interacts directly or indirectly with subunit 6 and mimics the otherwise artificially imposed genetic deletion of the subunit.

One possible candidate for such an intracellular signal may be the net positive charge on the cytochrome *c* side of the membrane, resulting from the proton motive force, which conceivably could titrate the multiple acidic residues on subunit 6 and thus modify the secondary structure of the subunit or cause it to reversibly dissociate from the bc_1 complex. Under conditions where oxidative phosphorylation is limited by the availability of ADP, the resulting increase in positive charge surrounding subunit 6 would step down the activity of the bc_1 complex and thus coordinate the rate of respiration with the need for ATP.

This proposed model for regulating the activity of the bc_1 complex by silencing half on the structural dimer (Fig. 12) may be unique to eucaryotic bc_1 complexes and may explain the role of one of the supernumerary polypeptides which are lacking in the purified bc_1 complexes from bacteria. However, although no counterpart of mitochondrial subunit 6 is found in the purified complexes from *P. denitrificans*, *Rhodospirillum rubrum*, *R. capsulatus*, or *R. sphaeroides*, the possibility cannot be ruled out that such a subunit exists in situ, but is lost during purification, especially since deletion of subunit 6 from the mitochondrial complex does not eliminate the cytochrome *c* reductase activity (133). It would be interesting to learn whether the bc_1 complexes of procaryotes are dimeric in situ.

Although deletion of subunit 6 does not interfere with respiration-dependent growth, we have constructed a mutant form of the subunit which gives rise to a petite phenotype (130). The wild-type chromosomal copy of *QCR6*, the nuclear gene encoding subunit 6, was replaced by a truncated copy capable of directing synthesis of a hybrid form of subunit 6, in which the acidic N-terminal presequence was replaced by a positively charged presequence. The resulting yeast strain, MES5, was unable to grow on nonfermentable carbon sources. Most surprising was the finding that this yeast strain lacked both cytochrome bc_1 and cytochrome *c* oxidase complexes, as indicated by a complete loss of cytochrome *c* reductase and oxidase activities, and loss of the spectrally detectable cytochromes of these two respiratory complexes (130).

MES5 is a nuclear petite and is not due to a spontaneously arising mutation in the mitochondrial genome (*rho*$^-$). MES5 spontaneously reverts, is complemented to wild-type respiration by a plasmid-encoded copy of *QCR6*, and yields a respiratory-competent diploid when mated against an *rho*^{0} tester strain. When MES5 is mated against a yeast strain in which the chromosomal copy of *QCR6* has been deleted, the resulting diploid has the same phenotype as the MES5 haploid strain. None of these criteria would be met if MES5 were a *rho*$^-$ mutant.

The mutant form of subunit 6 produced by MES5 must be interfering with some normal function of subunit 6, since the MES5 mutant is recessive. Transformation of the MES5 mutant with a wild-type copy of *QCR6* on a plasmid, or mating MES5 against a yeast carrying a wild-type chromosomal copy of *QCR6*, both give rise to a yeast which can respire. In other words, a normal copy of subunit 6 can overcome or block the damaging effect of the mutant subunit.

Two possible explanations for the MES5 phenotype come to mind. The first is that mitochondrial protein import is group specific and that the mutant form of subunit 6 blocks a step of mitochondrial protein import which is common to both the cytochrome bc_1 and cytochrome *c* oxidase complexes. Oligomycin-sensitive ATPase and mitochondrial protein synthesis are both unaffected in the MES5 mutant; thus, import of the proteins required for these is unaffected. Furthermore, a global blockage of mitochondrial protein import would be lethal. If this explanation is correct, one might expect to identify a new family of genes involved in mitochondrial protein import by isolating extragenic suppressors of the MES5 mutation.

The second possible explanation is that the cytochrome bc_1 and cytochrome *c* oxidase complexes are assembled through a common nucleation event and that they dissociate to form partially or completely assembled complexes at some point after subunit 6 of the bc_1 complex has been added. According to this model, assembly of the respiratory complexes would tolerate deletion of the subunit, but an altered copy would interrupt the assembly of the two complexes at a stage when they were part of a common precursor complex. If this explanation is correct, suppressors of the MES5 mutant might appear as mutations in other subunits of the bc_1 or oxidase complexes.

Subunits 7 and 8 of the yeast bc_1 complex are both intimately associated with cytochrome *b* (Fig. 2). Deletion of the gene for either of these subunits results in a petite phenotype in which cytochrome *b* is absent spectrally (102, 133). Deletion of the gene for either of the two subunits also results in a loss of both subunits. Analysis of the mRNA levels in these strains indicates that the pleiotropic effects resulting from the deletions are exerted at the posttranscriptional level, presumably owing to impaired assembly of the bc_1 complex.

Subunit 7 is apparently located on the matrix side of the membrane, by analogy with its counterpart in the beef heart complex (59). The protein has a molecular mass of 14.4 kDa, deduced from the sequence of the gene (37), and the N-terminal amino acid sequence of the mature protein is largely hydrophilic and is predicted to contain a high degree of α-helical structure (37).

Subunit 7, along with cytochrome *b*, is covalently labeled by an azido derivative of ubiquinone (185). If this subunit plays a role unique to eucaryotes in the redox reactions of ubiquinone, its topographic location would suggest that it is involved at ubiquinone-ubisemiquinone reducing center N. Japa et al. have reported that although subunit 7 is located on the matrix side of the membrane (73), it is involved in electron transfer at center P, which is generally accepted as being on the cytoplasmic side of the membrane. This hints at the possibility that the two quinone redox sites, center N and center P (Fig. 2), are not so distinctly separated. It may be possible to clarify the role of subunit 7 in ubiquinone reactions and ubisemiquinone stabilization by isolating and characterizing temperature-sensitive mutants.

Subunit 8 has a molecular mass of 12.3 kDa, calculated from the sequence of the gene and the N-terminal sequence of the mature protein (103). The protein is moderately hydrophobic, and the predicted secondary structure indicates that it consists predominantly of β-sheets, which may be arranged in five membrane-spanning sections to form a β-barrel structure. Subunit 8 has a low degree of sequence similarity to a 9.5-kDa protein of beef heart (18, 103), but the latter is predicted to be primarily α-helical (166). Because of the low degree of sequence similarity and differences in predicted secondary structure, it is not certain whether the yeast and beef heart proteins are homologous.

The nuclear gene (*QCR9*) encoding subunit 9 of the yeast

bc_1 complex has recently been cloned and sequenced (J. D. Phillips, M. E. Schmitt, J. D. Beckmann, and B. L. Trumpower, submitted for publication). If *QCR9* is deleted, the resulting yeast strain is petite, and ubiquinol-cytochrome *c* oxidoreductase activity of mitochondria from the deletion strain is less than 5% of that from the wild-type parent. Subunit 9 has a calculated molecular mass of 7.3 kDa, and if conservative amino acid substitutions are allowed it is 56% homologous to a subunit of similar molecular mass in the beef heart cytochrome bc_1 complex (129).

Subunit 9 contains a single possible transmembrane helix, and the amino acids which are conserved between the yeast and beef heart proteins are asymmetrically arranged along one face of the helix, implying that this domain of the protein is involved in a highly conserved interaction with another hydrophobic protein of the cytochrome bc_1 complex. Cytochromes *b* and c_1 are present in mitochondria from *S. cerevisiae* in which subunit 9 has been genetically deleted, but the iron-sulfur protein is absent and the optical spectrum of cytochrome *b* is altered in a manner which suggests that the integrity of heme ligation is compromised. The C terminus of subunit 9 contains a short, amphipathic helix and a short stretch of highly acidic residues. This subunit is proximal to cytochrome c_1 in the beef heart bc_1 complex (59, 166). We therefore speculate that subunit 9 interacts with iron-sulfur protein and cytochrome c_1 at the P side of the mitochondrial membrane and with cytochrome *b* in the hydrophobic, membrane interior (Fig. 2).

Genetics and Biogenesis of the Cytochrome bc_1 Complexes

The genes encoding the Rieske iron-sulfur protein (*fbcF*), cytochrome *b* (*fbcB*), and cytochrome c_1 (*fbcC*) are sequentially organized in an *fbc* operon, and there is a one-gene–one-protein relationship in *R. capsulatus* (34, 36, 56), *R. sphaeroides* (36), and *P. denitrificans* (92). The genes for the fourth, low-molecular-weight proteins which copurify with the *R. capsulatus* and *R. sphaeroides* bc_1 complexes (Fig. 10) have not been isolated. In *R. capsulatus* this gene is not part of the *fbc* operon (34, 54).

The mRNA transcribed from the *R. capsulatus fbc* operon starts approximately 240 base pairs upstream from a GTG triplet thought to be the start codon of the *fbcF* gene (56). GTG is also apparently used as the start codon in *P. denitrificans* (92). Two promoter consensus sequences for initiation of transcription, GCCGC, centered at base pair −10 and ACCCGTTGC, at base pair −35, were identified after S1 mapping and comparison with the mapped promoter of the *atp* operon of *Rhodopseudomonas blastica* (56). Portions of both of these consensus sequences are tandemly repeated in the *R. capsulatus* promoter region, but the significance of this repetition, if any, is not known.

Termination of transcription of the *R. capsulatus fbc* operon occurs approximately 170 bases downstream of the TGA stop codon of the *fbcC* gene. A double-looped secondary structure, with seven G · C pairs separating and stabilizing the two loops, encloses the 3′ end of the transcript and is thought to cause the transcript to release from the RNA polymerase (56).

The *fbc* operons appear to be constitutively expressed in *R. capsulatus* (34, 36, 56), *R. sphaeroides* (36), and *P. denitrificans* (92). The same 3.1-kilobase RNA species was detected when *R. capsulatus* was grown aerobically or photosynthetically (54), and an apparently identical bc_1 complex was purified from *P. denitrificans* grown under aerobic and denitrifying conditions (X. Yang, Ph.D. thesis, Dartmouth College, Hanover, N.H., 1988).

The genes encoding the bc_1 complex in *B. japonicum* are also organized in an operon, but it is unusual in that only two genes, *fbcF* and *fbcH*, encode the three redox proteins (146). The open reading frame of *fbcF* corresponds to a protein of molecular weight 18,791 having a high degree of homology to the Rieske iron-sulfur proteins from *P. denitrificans*, *R. capsulatus*, and *S. cerevisiae*. The open reading frame of *fbcH* corresponds to a protein of molecular weight 76,469. Alignment of sequences revealed that the N-terminal half of the protein predicted from *fbcH* had a high degree of sequence similarity to *R. capsulatus* cytochrome *b*, whereas the C-terminal half of the predicted *fbcH* protein was similar to cytochrome c_1. The region of the DNA sequence in *fbcH* corresponding to the junction between the two cytochromes did not contain stop or start codons or any ribosomal binding site. It was therefore concluded that the *fbcH* gene encoded both cytochromes *b* and c_1 (146).

Antibodies raised against cytochrome *b*, cytochrome c_1, and the Rieske iron-sulfur protein of *P. denitrificans* reacted specifically with three polypeptides in membranes of *B. japonicum* (M. E. Schmitt, X. Yang, and B. L. Trumpower, unpublished observations), and covalently bound heme was found in a protein of molecular weight approximately 28,000, as expected if cytochrome c_1 is a separate protein. No protein of the size of the *b*-c_1 fusion protein expected from an unprocessed *fbcH* gene product could be detected by the antibodies (146). These findings led to the conclusion that the *fbcH* gene product is posttranslationally processed, so that cytochromes *b* and c_1 are separate proteins. This is apparently the first instance in bacteria in which a single gene codes for two distinct proteins (146).

The four genes encoding the four subunits of the *bf* complex in *Nostoc* spp. are clustered in two widely separated operons, *petCA* and *petBD* (78, 79). The genes for the iron-sulfur protein, *petC*, and cytochrome *f*, *petA*, are cotranscribed as a 2-kilobase mRNA, whereas the genes for the two subunits of cytochrome *b*, *petB* and *petD*, are cotranscribed as a 1.8-kilobase mRNA. The last two genes lack introns, in contrast to the chloroplast and mitochondrial genes for cytochrome *b*, which contain numerous introns (154). All four genes appear to be present in single copy, indicating that one form of *bf* complex functions in *Nostoc* spp. grown under various conditions (78).

In eucaryotes containing mitochondria the gene for cytochrome *b* is encoded in that organelle (for a review, see reference 154), whereas in plants the two genes for the two subunits of cytochrome *b* and that for cytochrome *f* are encoded in the chloroplast genome (2, 71, 175). The remaining subunits, including the iron-sulfur protein and the supernumerary polypeptides, are encoded in the nucleus in all eucaryotes.

In *S. cerevisiae* the eight nuclear genes of the bc_1 complex are randomly distributed throughout the 16 yeast chromosomes, and there appears to be only one copy of each gene. The expression of some, and perhaps all, of the nuclear genes of the bc_1 complex is coordinately controlled by heme and oxygen at the level of transcription, the latter effector acting through the former (53, 111). Like other proteins which are not required for fermentative growth, some, and perhaps all, of these genes are repressed when *S. cerevisiae* is grown on high concentrations of glucose (49, 101). However, the effects of oxygen and carbon source on transcription and translation have not been investigated for all of the bc_1 complex genes.

The mechanism by which oxygen and carbon source coordinately regulate the expression of a nuclear gene for a

respiratory protein is best understood for *CYC1*, the nuclear gene encoding cytochrome *c* (53). As far as is known, the effects of oxygen on *CYC1* expression are mediated solely through heme. Four regulatory genes, *HAP1* through *HAP4*, acting through two upstream activating sequences, UAS1 and UAS2, regulate *CYC1* expression. *HAP1* encodes a heme-dependent DNA-binding protein which activates UAS1. *HAP2*, *HAP3*, and *HAP4* encode three subunits of a heteromeric regulatory complex, which binds to UAS2 and activates *CYC1* transcription 50-fold when yeast cells are switched from glucose to a nonfermentable carbon source (53, 64).

Transcription of *CYT1*, the gene for cytochrome c_1, is regulated by *HAP1* through *HAP4* via UAS1 and UAS2 in a manner similar to *CYC1* (53). *COR1*, which codes for core protein 1, is also regulated by heme (111), presumably through the *HAP1* through *HAP4* system, demonstrating that heme also regulates the synthesis of respiratory proteins which do not contain heme. Synthesis of the β subunit of the mitochondrial ATP synthase was not regulated by heme (111).

Maarse et al. (101) noted that the genes for core proteins 1 and 2, iron-sulfur protein, 17-kDa subunit 6, 14-kDa subunit 7, and 11-kDa subunit 8 all contain the UAS2 consensus sequence TNPuTTGGT (115), but the expression of these bc_1 complex genes in *hap1* through *hap4* mutant strains has not been reported. Attempts to demonstrate transcriptional factor binding to the UAS2 consensus sequence in the 11-kDa subunit 8 gene promoter were unsuccessful (102). Whether this consensus sequence is present in the promoter of *QCR9* is not known.

Transcription of the genes for core protein 2, *COR2* (116), cytochrome c_1, *CYT1* (53), the iron-sulfur protein, *RIP1* (101), subunit 6, *QCR6* (157), subunit 7, *QCR7* (157), subunit 8, *QCR8* (157), and subunit 9, *QCR9* (T. A. Brown, J. D. Phillips, and B. L. Trumpower, unpublished observations), are all repressed by glucose. Although the 5′-flanking regions of most of the genes contain the consensus sequence UAS2, it is not known whether this regulatory element, alone or in combination with others, mediates carbon source regulation of transcription.

Although the only known regulation of expression of the nucleus-encoded subunits of the bc_1 complex is at the level of transcription, we suspect there might be an additional mechanism of regulation at the level of mRNA splicing. The nuclear gene for the 7.3-kDa subunit 9 (*QCR9*) of the yeast bc_1 complex contains a 213-base-pair intron. Since introns are rare in *S. cerevisiae* nuclear genes (52), the retention of the intron suggests that it serves some function. In addition, the *QCR9* intron contains a nucleotide sequence, located upstream from the consensus TACTAAC splicing sequence, in which 15 of 18 and 21 of 28 nucleotides are identical to a sequence within the intron of *COX4* (131), the nuclear gene encoding cytochrome *c* oxidase subunit 4. This sequence was not found in any of the other yeast nuclear gene introns. Experiments are now in progress to test whether these two introns may be targets for coordinate regulation of the cytochrome bc_1 and cytochrome *c* oxidase complexes.

The nucleus-encoded subunits of the bc_1 and *bf* complexes are synthesized in the cytoplasm and posttranslationally imported into the mitochondria or chloroplast. The mechanisms of posttranslational import and processing of nucleus-encoded mitochondrial and chloroplast proteins are the subjects of intense investigation, and numerous excellent reviews are available (7, 60, 66, 120, 162). According to the generalized model for mitochondrial protein import, cytoplasmically synthesized mitochondrial proteins contain a targeting presequence, which is removed posttranslationally in one or two steps by proteases located in the matrix or inner mitochondrial membrane. These targeting presequences vary in length, but typically are positively charged, somewhat enriched in serine and threonine residues, and capable of forming amphipathic helices (126, 127, 163).

Without attempting to duplicate other reviews on biogenesis of mitochondrial proteins, I would like to point out that posttranslational processing and import of many of the nucleus-encoded subunits of the cytochrome bc_1 complex do not conform to the generalized model for import of proteins from the cytoplasm into the mitochondria. Of the eight nucleus-encoded proteins of the cytochrome bc_1 complex, core proteins 1 and 2, cytochrome c_1, and the iron-sulfur protein are imported and posttranslationally processed according to this model (66, 67). However, the import and posttranslational processing of the remaining four nucleus-encoded subunits do not conform to this model. Three of these proteins, subunits 7, 8, and 9, have only the N-terminal methionine removed posttranslationally. These subunits thus lack a cleavable presequence, and whether there are amphipathic targeting sequences within the N-terminal regions of the mature proteins is not established. The lack of a cleavable presequence does not correlate with any one topographical location, since subunits 7, 8, and 9 appear to be located on the matrix surface, in the hydrophobic interior of the complex, and on the cytoplasmic surface, respectively (Fig. 2).

Subunit 6 does contain a 25-amino-acid presequence which is posttranslationally removed and which is amphipathic, but this sequence is enriched in acidic amino acids and contains only one positively charged residue and only one threonine (130). Examination of the sequence of the remainder of the protein fails to reveal any amphipathic region of positively charged residues. The highly acidic subunit 6, which we propose regulates the activity of the dimeric bc_1 complex by a reversible dissociation from the complex (Fig. 12), appears to be targeted to the inner mitochondrial membrane by a different mechanism than the generalized model described above.

Mutational analysis of the iron-sulfur protein has revealed another aspect of mitochondrial protein processing which merits further investigation. Using a yeast strain from which the iron-sulfur protein gene (*RIP1*) was deleted and in vitro mutagenesis of the cloned gene, we have identified numerous temperature-sensitive mutations which completely block protease processing of the iron-sulfur protein at the nonpermissive temperature (10, 11; L. A. Graham and B. L. Trumpower, unpublished observations). These single-amino-acid changes are distributed throughout the mature part of the protein. One possible explanation for this family of mutations is that the amino acid changes alter the energetics of protein unfolding (48), thus blocking import of the precursor form of the iron-sulfur protein.

We have also replaced each of the conserved cysteines and histidines in the iron-sulfur protein (Fig. 5) by site-directed mutagenesis and found that each of these mutant iron-sulfur proteins was processed to the mature size at the nonpermissive temperature, but was nonfunctional (Graham and Trumpower, unpublished). This suggests either that the iron-sulfur cluster is added to the protein after the last protease-processing step or that lack of iron-sulfur cluster

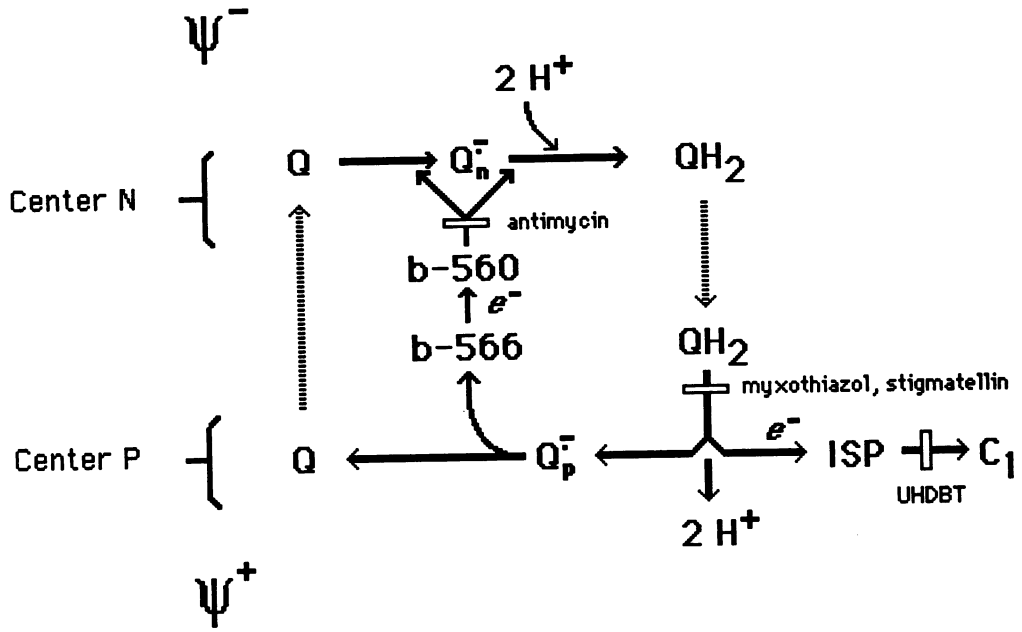

FIG. 13. Proton motive Q-cycle pathway of electron transfer and mechanism of proton translocation in the cytochrome bc_1 complex. The scheme depicts electron transfer from ubiquinol (QH_2) to cytochrome c_1 through redox components of the complex and shows the topographical disposition of reactions between the electronegative N side and electropositive P side of the membrane. Centers N and P correspond to centers *i* and *o*, previously used to designate these sites as inside and outside the inner mitochondrial membrane. Center N also corresponds to the quinol reductase site, and center P corresponds to the quinol oxidase site. Under pre-steady-state conditions, ubiquinol is oxidized by the iron-sulfur protein (ISP) at center P in a reaction which can be inhibited by myxothiazol, stigmatellin, or UHDBT. Oxidation of ubiquinol releases $2H^+$ at the P side of the membrane and generates a low-potential ubisemiquinone anion (Q_p^-), which immediately transfers one electron to cytochrome *b*-566. This electron is then transferred against the electrochemical potential inward through the membrane from the cytochrome *b*-566 heme to the *b*-560 heme. Cytochrome *b*-560 reduced by this route is alternately reoxidized by either ubiquinone (Q) or ubisemiquinone anion (Q_n^-) in an antimycin-sensitive reaction at center N. Reduction of ubisemiquinone anion (Q_n^-) at center N consumes $2H^+$ at the N side of the membrane. If formation of ubisemiquinone anion (Q_p^-) at center P is blocked by inhibitors, or by prereduction or removal of the iron-sulfur protein, the *b* cytochromes can be reduced by reversal of the antimycin-sensitive pathway at center N. Symbols: . . . . . ., prerequisite movement of ubiquinol and ubiquinone between the ubiquinol oxidase site (center P) and the ubiquinone/ubisemiquinone reductase site (center N); □, electron transfer reactions blocked by the inhibitors.

insertion does not block the protease processing of the apoprotein.

MECHANISM OF ELECTRON TRANSFER AND ENERGY TRANSDUCTION

The Proton Motive Q Cycle

Electrons are transferred from ubiquinol to cytochrome *c* through the cytochrome bc_1 complex by a mechanism known as the proton motive Q cycle (110). The Q cycle describes the sequential pathway of electron transfer among the redox prosthetic groups of the bc_1 complex and accounts for the linkage of proton translocation to electron transfer. A diagram of the Q-cycle mechanism is shown in Fig. 13. At the risk of oversimplification, I will first describe the Q cycle in stepwise fashion. I will then discuss some nuances and implications of the Q cycle mechanism and summarize some of the experimental evidence in support of the mechanism.

Two electrons are transferred from ubiquinol, one at a time, through the cytochrome bc_1 complex to reduce two molecules of cytochrome *c*. For the net oxidation of one molecule of ubiquinol and reduction of two molecules of cytochrome *c*, four protons are deposited on the outer side of the inner mitochondrial membrane or bacterial plasma membrane, and two protons are consumed on the inner side of the membrane as ubiquinone is rereduced to ubiquinol by a dehydrogenase, hydrogenase, or photosynthetic reaction center (Fig. 1). This transmembrane, proton-moving electron circuit establishes a proton gradient across the membrane. In mitochondria the polarity of the Q cycle is such that the outer surface of the inner membrane is positive relative to the matrix. In bacteria the external surface of the plasma membrane is positive relative to the cytoplasm.

In the first step of the Q cycle ubiquinol is oxidized by transfer of one electron to the iron-sulfur protein to form a ubisemiquinone anion (Fig. 13). This oxidation deposits two protons on the positive side of the membrane. One is released coincident with oxidation of ubiquinol to ubisemiquinone; the second is released essentially simultaneously with the first, as ubisemiquinone ionizes to ubisemiquinone anion. This ubisemiquinone anion immediately reduces the low-potential cytochrome *b*-566 heme.

As a result of this oxidation reaction at the positive surface of the membrane, the two electrons from ubiquinol diverge. One is transferred to the iron-sulfur protein and then onto cytochrome c_1. This electron is then transferred to a terminal oxidase or photosynthetic reaction center, via one of the *c* cytochromes (Fig. 1). The second electron, transferred from ubisemiquinone to cytochrome *b*-566, "recycles" through the bc_1 complex as it is transferred from the low-potential *b*-566 to the higher-potential *b*-560 (Fig. 13). Cytochrome

TABLE 3. Mutations in cytochrome *b*

Amino acid change[a]	Phenotype	Reference
Ile-17 to Phe	Diuron resistant	41, 43
Asn-31 to Lys	Diuron resistant	41, 43
Asn-31 to Lys	Antimycin resistant	30
Gly-37 to Val	Antimycin resistant	41
Ala-37 to Val	Antimycin resistant	169
Ala-37 to Gly	Diuron resistant	169
Phe-129 to Leu	Myxothiazol resistant	42
Cys-133 to Tyr	*b*-566 diminished	Abstract[b]
Gly-137 to Arg	Myxothiazol and strobilurin resistant	42
Gly-143 to Asp	Nonfunctional Qz site	35
Ile-147 to Phe	Stigmatellin resistant	42
Thr-148 to Ala	Stigmatellin resistant	35
Leu-198 to Phe	Funiculosin resistant	A. Colson, personal communication
Ser-206 to Leu	*b*-562 modified	Abstract[b]
Met-221 to Leu	High-affinity antimycin binding lost	Abstract[b]
Phe-225 to Leu	Diuron resistant	41, 43
Phe-225 to Ser	Diuron resistant	41, 43
Lys-228 to Met	Antimycin resistant	41
Lys-228 to Ile	Antimycin resistant	30
ΔLeu-230, Ile-231, Thr-232/Ser	Antimycin resistant	30
Asn-256 to Tyr	Myxothiazol and strobilurin resistant	42
Leu-275 to Ser	Myxothiazol and strobilurin resistant	42
Leu-275 to Phe	Myxothiazol and strobilurin resistant	42
Leu-275 to Thr	Myxothiazol resistant	42
Leu-282 to Phe	High-affinity antimycin binding lost	Abstract[b]
Val-291 to Ala	Stigmatellin resistant	35
Gly-340 to Glu	High-affinity antimycin binding lost	Abstract[b]

[a] The numbering of amino acids is for the *S. cerevisiae* cytochrome *b*, shown in Fig. 3. Mutations obtained in *Kluyveromyces lactis* and *R. capsulatus* have been assigned the number of the corresponding amino acid in *S. cerevisiae*.
[b] D. Meunier-Lemesle, J. P. Di Rago, and P. Chevillotte-Brivet, Abstr. 5th Eur. Bioenerg. Conf. 1988, p. 76.

b-560 then reduces ubiquinone to ubisemiquinone anion at the negative surface of the membrane.

As a result of the preceding series of reactions, one molecule of ubiquinol is oxidized, one electron is transferred to cytochrome c_1 en route to cytochrome *c* and the terminal oxidase or photosynthetic reaction center, a ubisemiquinone anion ($Q_n^{\cdot-}$) is formed at the negative side of the membrane, and two protons are deposited on the positive side of the membrane. At this point the Q cycle is only half complete. A second molecule of ubiquinol is then oxidized by the iron-sulfur protein, transferring one electron to cytochrome c_1 and one electron to cytochrome *b-566* and then to *b*-560 and depositing two more protons on the positive side of the membrane. Cytochrome *b*-560 then reduces the previously formed ubisemiquinone anion ($Q_n^{\cdot-}$) to ubiquinol at the negative surface of the membrane, consuming two protons from the negative side of the membrane and completing the Q cycle.

The sum of these reactions is that two molecules of ubiquinol are oxidized to ubiquinone, one molecule of ubiquinol is formed by rereduction of one of these ubiquinones, two molecules of cytochrome *c* are reduced, four protons are deposited on the positive side of the membrane, and two protons are consumed from the negative side of the membrane. One complete Q-cycle sequence requires that the redox proteins of the bc_1 complex, the iron-sulfur protein, cytochrome c_1, and the *b* cytochromes, undergo two "redox turnovers." In the course of one Q-cycle sequence, cytochrome *b*-560 alternately reduces ubiquinone to ubisemiquinone anion and ubisemiquinone anion to ubiquinol (Fig. 13).

There are several inhibitors, isolated from microorganisms, which specifically inhibit electron transfer through the cytochrome bc_1 complex. To date, all of these inhibitors can be grouped into one of three classes, depending on their site of action (165). The class I inhibitors, also referred to as center P inhibitors and exemplified by myxothiazol and stigmatellin, block the oxidation of ubiquinol by the iron-sulfur protein (Fig. 13). These bind to cytochrome *b*, as indicated by the fact that all of the known mutations conferring resistance to the inhibitors in *S. cerevisiae* map to the cytochrome *b* gene (Table 3). In addition, myxothiazol and stigmatellin both shift the optical spectrum of *b*-566 (167, 168).

However, the center P inhibitors also affect the iron-sulfur protein. Stigmatellin increases the midpoint potential of the iron-sulfur cluster by 540 mV (168), and myxothiazol appears to displace the quinone inhibitor UHDBT from the iron-sulfur protein (167). Brandt et al. (23) have shown that inhibition by myxothiazol is noncompetitive with respect to ubiquinol. As discussed above, I interpret these findings to indicate that the center P-binding sites of myxothiazol and UHDBT overlap, that this site is composed of structural domains from the iron-sulfur protein and cytochrome *b*, and that it is normally occupied by the ubiquinone product formed by the center P oxidation of ubiquinol.

Class II inhibitors are the hydroxyquinones, such as UHDBT (20). UHDBT inhibits electron transfer at center P, and the site where UHDBT binds apparently overlaps that where myxothiazol binds, since the latter displaces the former (167). As discussed above, it appears that UHDBT binds to the iron-sulfur protein and cytochrome *b* at a site otherwise occupied by ubiquinone, to which UHDBT is structurally similar. To date no yeast mutants resistant to UHDBT have been isolated.

The third class of inhibitors, such as antimycin, blocks electron transfer between cytochrome *b*-560 and ubiquinone. Antimycin binds to cytochrome *b*, proximal to the *b*-560

heme (12). The antimycin-binding site is also probably proximal to the Q_n^- reaction site, since antimycin abolishes the stable ubisemiquinone radical signal (107, 114). All of the mutations conferring antimycin resistance in *S. cerevisiae* have mapped to the cytochrome *b* gene (Table 3).

The preceding description, although formally correct, omits numerous important nuances and implications of the Q cycle. The redox proteins of the bc_1 complex must be asymmetrically arranged in the membrane, with the iron-sulfur protein and cytochrome c_1 on the positive surface. Cytochrome *b* must be transmembranous, so that the two *b* hemes constitute a transmembrane electron circuit. Structural studies on the bc_1 complexes of mitochondria suggest that the redox proteins are so arranged (Fig. 2).

The divergent oxidation of ubiquinol at center P transfers one electron to the iron-sulfur protein at a redox potential of approximately +250 mV and a second electron to cytochrome *b*-566 at a redox potential of approximately −100 mV. The ubiquinol-ubisemiquinone anion and ubisemiquinone anion-ubiquinone redox couples at center P thus differ in redox potentials by about 350 mV, which indicates that the intermediate ubisemiquinone anion (Q_p^-) is unstable. The relationship between the stability of ubisemiquinone anion and the midpoint potentials of the ubisemiquinone anion couples has been discussed previously (13, 110, 149).

The reduction of ubiquinone and ubisemiquinone anion by cytochrome *b*-560 must take place at similar potentials, assuming that the potential of *b*-560 can change at most 100 mV coincident with reduction of ubiquinone to ubiquinol (128). The ubiquinol-ubisemiquinone anion and ubisemiquinone anion-ubiquinone redox couples at center N must therefore have similar redox potentials, which indicates that the intermediate ubisemiquinone anion (Q_n^-) must be relatively stable. A thermodynamically stable ubisemiquinone, having properties consistent with those expected of Q_n^-, has been found in the bc_1 complexes of mitochondria (114) and *P. denitrificans* (107).

If the two heme groups of cytochrome *b* form a transmembranous circuit, recycling of the electron through the *b* cytochromes would be opposed by the membrane potential, providing the basis for respiratory control by the proton motive force in the bc_1 complex. Conversely, an applied potential should be capable of driving electron transfer from the high-potential *b*-560 to the lower-potential *b*-566. West et al. (172) demonstrated electron conduction between the two *b* hemes, driven by an applied potential.

The stoichiometry of four protons deposited to the positive side of the membrane per pair of electrons transferred to two molecules of cytochrome *c* depends on the divergence of the two electrons from ubiquinol oxidation and the recycling of the one electron through the *b* cytochromes. If the second electron from ubiquinol follows the thermodynamically preferred route, that is, if the low-potential ubisemiquinone anion (Q_p^-) reduces iron-sulfur protein rather than *b*-566, the resulting short circuiting of the cycle would lower H^+/e^- from 2 to 1. The *b*-566 domain of cytochrome *b* and the iron-sulfur protein must therefore interact in a manner which provides a significant kinetic advantage to ubisemiquinone anion (Q_p^-)/*b*-566 electron transfer, to counteract a 350-mV increment in potentials which otherwise favors reduction of iron-sulfur protein by the strongly reducing ubisemiquinone anion.

All of the experimental observations on electron transfer and proton translocation through the cytochrome bc_1 complex are consistent with a proton motive Q-cycle mechanism. Hereafter I will describe the observations which, in my view, have been important in establishing the Q-cycle mechanism, and which distinguish that mechanism from others. Mitchell first proposed the Q-cycle mechanism (109, 110) and incorporated the novel suggestion of Wikström and Berden (174) that the two electrons from ubiquinol oxidation diverge, one reducing cytochrome *b* and the other reducing cytochrome c_1. Following Mitchell's proposal the Q cycle was modified to incorporate the role of the iron-sulfur protein and to specify the sequence of the divergent ubiquinol oxidation at center P (147).

The Q cycle accounts for the participation of all of the redox proteins of the bc_1 complex in electron transfer through the complex, is consistent with the kinetics of reduction and reoxidation of the cytochromes of the complex (33), and uniquely explains how cytochrome *b*-566, which has a midpoint potential approximately 150 mV more negative than that of the ubiquinol-ubiquinone couple, can participate in the oxidation of this couple. The Q cycle also accounts for the observation, discovered and extensively documented by Chance (29), that a rapid pulse of oxygen causes the expected oxidation of cytochrome c_1, but induces an unexpected reduction of cytochrome *b*. This transient, oxidant-induced reduction of cytochrome *b* results from the rapid generation of a low-potential reductant for cytochrome *b* (Q_p^-), linked to c_1 (and iron-sulfur protein) oxidation, and the relatively low rate of *b*-560 reoxidation (Fig. 13).

Much of the evidence supporting the Q cycle was gathered from examining the pre-steady-state reduction of cytochromes *b* and c_1 in mitochondria. One such example is the triphasic nature of cytochrome *b* reduction (74). When electrons are delivered to the fully oxidized cytochrome bc_1 complex from ubiquinol, cytochrome c_1 is reduced in an essentially linear manner. During the initial phase of c_1 reduction, cytochrome *b* is also partially reduced. This is then followed by reoxidation and then by extensive rereduction of the *b*.

Within the context of the Q cycle (Fig. 13), the interpretation of the triphasic *b* reduction is that the initial phase of *b* reduction occurs by oxidation of ubiquinol at center P in a reaction which is linked to c_1 reduction through the iron-sulfur protein. This reaction is inhibited by myxothiazol, but not by antimycin (142). Cytochrome *b* is then reoxidized in an antimycin-sensitive reaction as ubiquinone and ubisemiquinone are generated at center N (14). The third phase, in which cytochrome *b* is more slowly and more extensively reduced, proceeds by a reversal of the center N reactions, with ubiquinol and ubisemiquinone alternately reducing *b*-560 in an antimycin-sensitive reaction (142). This reversal of the center N reactions, which presumably occurs only under pre-steady-state conditions, results from iron-sulfur protein and c_1 being reduced during the first phase of reduction and thus being unavailable to continue oxidizing ubiquinol. The same antimycin-sensitive reduction of the *b* cytochromes through center N can be demonstrated by prereducing c_1 and iron-sulfur protein with ascorbate before reducing the *b* cytochromes with ubiquinol (153).

Two additional observations provide strong support for the Q-cycle mechanism. Both demonstrate that there are two pathways for cytochrome *b* reduction, through center P and center N, under pre-steady-state conditions. The first is the finding that removal of the iron-sulfur protein from the cytochrome bc_1 complex prevents reduction of cytochrome c_1, and thus prevents reduction of cytochrome *b* through center P, but allows reduction of cytochrome *b* through center N (47, 150). The latter reaction is antimycin sensitive (47). Likewise, removal of the iron-sulfur protein eliminates

the oxidant-induced reduction of cytochrome *b*, since the linkage between c_1 oxidation and ubisemiquinone generation at center P (Fig. 13) is broken (21). All of these iron-sulfur protein-dependent reactions are restored when the iron-sulfur protein is reconstituted to the complex (148, 151).

The second type of evidence for two routes of cytochrome *b* reduction under pre-steady-state conditions is the finding by Von Jagow and Link (165) that a new class of microbial toxins, the class I inhibitors described above, block cytochrome *b* reduction through an antimycin-insensitive pathway. Members of this class of inhibitors act at center P, and include myxothiazol (165, 167), strobilurin (165), and stigmatellin (168). When added alone, antimycin does not inhibit the pre-steady-state reduction of cytochrome c_1 or cytochrome *b*, which occurs via center P (22). Likewise, the center P inhibitors, which block c_1 reduction, do not block *b* reduction through center N (167). However, when antimycin is added together with myxothiazol or strobilurin, the combined inhibitors block both cytochrome *b* and c_1 reduction (167). This double-kill effect is due to simultaneous blockage of electron transfer through both centers N and P (Fig. 13).

These findings provide compelling evidence for the proton motive Q-cycle mechanism, and there is no other mechanism which accounts for all of these findings. Although the Q cycle was first established for the mitochondrial bc_1 complexes, subsequent tests of the mechanism in *P. denitrificans* indicate that it is operative in procaryote bc_1 complexes as well (181). However, it remains to be tested whether the Q cycle is universal and, in particular, whether this mechanism appeared coincident with the cytochrome bc_1 complex in evolution.

I can envision a more primitive electron transfer mechanism in which both electrons from ubiquinol oxidation are transferred to the iron-sulfur protein and c_1, rather than following divergent routes with recycling of one electron through cytochrome *b*. Such a mechanism, involving a simple transmembranous reduction and reoxidation of ubiquinone and ubiquinol, would drop H^+/e^- from 2 to 1, but this lower efficiency of energy transduction may be adequate to support primitive microorganisms, especially since bacteria can grow at a reduced rate while bypassing the bc_1 complex (see above). If such a primitive mechanism exists, I would expect it most probably to be in the electron transfer pathway in *Chlorobium* spp., the mechanism of which remains to be investigated.

Quinone Redox Sites in the Cytochrome bc_1 Complex

There must be two sites for oxidation-reduction of the ubiquinol-ubiquinone couple in the bc_1 complex. These two sites must communicate asymmetrically with opposite surfaces of the membrane, they must be electronically insulated from each other, and they must confer distinctly different properties on the intermediate ubisemiquinone anion. These two quinone redox sites must be on cytochrome *b*, since the three-subunit bc_1 complex of *P. denitrificans* transfers electrons by a Q-cycle mechanism (181) and exhibits a stable ubisemiquinone anion (107), although it is possible that one or more of the supernumerary polypeptides contribute to the quinone redox sites in mitochondria (see above).

Since Q_n^- is stabilized by approximately 10 orders of magnitude relative to ubisemiquinone in solution (110), there must be one or more amino acid residues on cytochrome *b* which facilitate preferential binding of ubisemiquinone anion, relative to ubiquinone and ubiquinol. Binding of the substituents on the quinone ring to enhance a more planar geometry may also contribute to ubisemiquinone anion stabilization (149). The amino acid residues which form the Q_n^- site must direct protonation of ubiquinone from the N side of the membrane, and also be sufficiently proximal to the *b*-560 heme group to permit redox exchange between the *b*-560 heme group and both the ubiquinone-ubisemiquinone anion couple and ubisemiquinone anion-ubiquinol couple at center N.

On the basis of the location of the antimycin-resistant mutations in cytochrome *b*, the topography of the transmembrane helices, and the location of the histidine ligands to the heme of *b*-560 (Fig. 3), I propose that the Q_n^- site is formed by the terminal regions of helices 5 and 6, the connecting loop between these two helices, and the N-terminal region of helix 1. It is notable that the chloroplast cytochrome b_6 is divided into two subunits at the junction between helices 5 and 6 (Fig. 3). The elimination of this continuity may account for the lack of antimycin sensitivity in the chloroplast *bf* complex and the failure to date to demonstrate a stable ubisemiquinone radical analogous to Q_n^- in the chloroplast *bf* complex.

The ubiquinol oxidase site at center P must direct the divergent oxidation of ubiquinol and ubisemiquinone anion by iron-sulfur protein and cytochrome *b*-566, as noted above, and must direct the deprotonation of ubiquinol to the P side of the membrane. Rich (123) has pointed out that removal of the first electron from ubiquinol is facilitated markedly by prior ionization to ubiquinol anion. Consequently, center P must contain one or more amino acids capable of promoting deprotonation, most probably through hydrogen bonding. In addition, the amino acids surrounding the center P site must prevent access of water to this strongly reducing ubisemiquinone and must prevent it from escaping, otherwise the semiquinone anion would react to form hydroxyl or oxygen radicals or would reduce cytochrome *c*.

The mutations in cytochrome *b* which confer resistance to center P inhibitors (Table 3) are located in the termini of helices 3, 7, and 8 and in the hydrophilic helix 4 and the extended hydrophilic PEWY loop between helices 6 and 7 (Fig. 3). The latter region also contains a large number of the conserved protonic amino acids on the electropositive side of the membrane. It seems likely that the terminal regions of helices 7 and 8 abut the corresponding terminus of helix 3, that the hydrophobic domain provided by these three helices is capped by the N-terminal portion of helix 4 and the hydrophilic PEWY loop, and that these domains in cytochrome *b*, together with the conserved C-terminal region of the iron-sulfur protein (Fig. 5), form center P.

In my view, it is not necessary that there be two distinct binding sites for the redox forms of ubiquinone. Rather, it seems possible that ubiquinone binds at one region on cytochrome *b* in the median of the membrane and that the quinone ring reacts alternately at centers N and P by rotation from this single location. This may explain the observation that perturbation of the matrix-disposed subunit 7 of the *S. cerevisiae* bc_1 complex interferes with ubiquinol oxidation at center P (73). There is no evidence to establish whether two molecules of ubiquinone, ubisemiquinone, or ubiquinol can simultaneously occupy center N and center P.

Proton Conduction Pathways on Cytochrome *b*

The quinone-quinol-semiquinone reaction sites at both center N and center P must exclude oxygen, but they must permit deprotonation-protonation linked to oxidation-reduc-

TABLE 4. Conserved protonic amino acids in *S. cerevisiae* cytochrome *b*[a]

Amino acid	Helix location	Topographical location
Asp-71	2	Center P
Arg-79	2	Membrane interior
Ser-87	2	Membrane interior
Lys-99	2	Center N
Tyr-132	3	Membrane interior
Thr-145	4	Center P
Thr-148	4	Center P
Asp-160	4	Center P
Thr-175	4–5	Center P
Arg-178	5	Center P
His-202	5	Center N
Asp-229	6	Membrane interior
Glu-261	6–7	Center P
Thr-265	6–7	Center P
Glu-272	6–7	Center P
Arg-283	6–7	Center P
Ser-140	3–4	Center P
Ser-206	5–6	Center N
His-253	6–7	Center P
Asp-255	6–7	Center P

[a] The table lists those protonic amino acids, glutamate, aspartate, serine, threonine, arginine, lysine, histidine, glutamine, asparagine, and tyrosine, which are conserved among the 25 cytochrome *b*'s which have been sequenced to date. Serine 140, serine 206, histidine 253, and aspartate 255 are not conserved in cytochrome b_6. In identifying conserved residues from alignments of the sequences, exchange of glutamate for aspartate, serine for threonine, lysine for arginine, and glutamine for asparagine have been considered to be conservation of sequence. The conserved protonic amino acids are underlined in Fig. 3 and are identified in this table as residing in one of the helices, or in the connecting span between two helices.

tion of the ubiquinol-ubisemiquinone anion couple. This requires that there be two proton conduction pathways, presumably composed of protonic amino acids, which link the anhydrous center N and center P redox sites with the aqueous membrane interface. These proton-conducting residues are most probably on cytochrome *b*, and should be highly conserved.

In Table 4 I have enumerated the protonic amino acids which are conserved among the cytochrome *b*'s which have been sequenced to date. The location of these potential proton conductors in the *S. cerevisiae* cytochrome *b* is shown in Fig. 3. There are only two conserved protonic amino acids in the vicinity of the postulated center N, lysine 99 and histidine 202. Serine 206 is partly conserved, except in chloroplasts. This suggests that proton conduction to center N may occur through bound water. In this regard it is interesting that this surface of the *N. crassa* bc_1 complex appears to contain an aqueous cleft (95).

There are a relatively large number of conserved protonic residues at center P, and these are distributed between the PEWY loop and the hydrophilic helix 4. Beattie and coworkers (D. Beattie, personal communication) have recently established that aspartate 160 is covalently labeled by *N,N'*-dicyclohexylcarbodiimide (DCCD) coincident with inhibition of proton motive activity of the *S. cerevisiae* bc_1 complex. This residue is not universally conserved and is notably absent from the bc_1 complex of *P. denitrificans*, which is not sensitive to such inhibition by DCCD (181). Identifying the proton-conducting pathways on cytochrome *b* which link the redox and acid-base chemistry of ubiquinone is one of the more challenging questions to be answered to complete our understanding of the mechanism of energy transduction in the cytochrome bc_1 complex.

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